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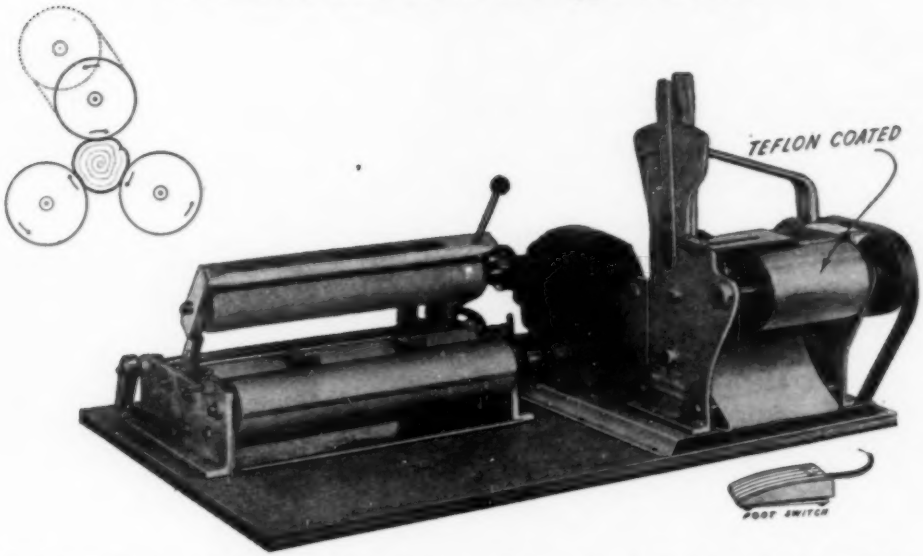

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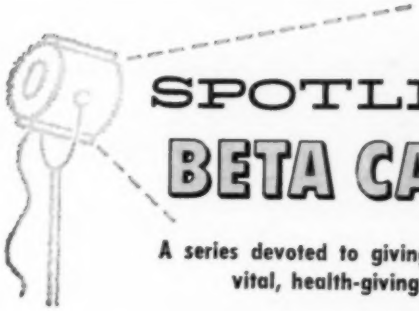

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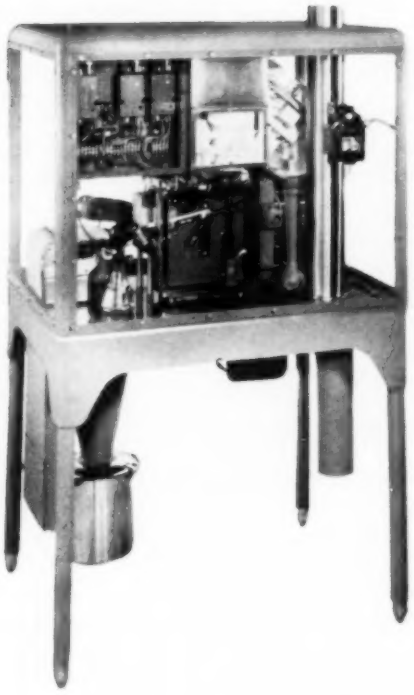

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CEREAL CHEMISTRY

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THE PROTEINS IN WATER EXTRACTS OF CORN[1]

E. M. CRAINE AND K. E. FAHRENHOLTZ[2]

ABSTRACT

The proteins in water extracts of corn form insoluble precipitates while aging at low ionic strengths and when frozen and thawed. This instability prevents assay of protein fractions by solubility methods. The extracts are stabilized by addition of salt or by removal of anion contaminants.

Some of the extract proteins react with phytate ions at pHs below 5 to form insoluble complexes. Precipitation of these complexes aids the fractionation of the proteins. Increasing the ionic strength of the extracts decreases the pH at which a particular protein-phytate complex precipitates.

Among six protein components identified electrophoretically, three are major portions of the protein. Each can be prepared in fairly homogeneous condition. At least one of these combines with a dialyzable anion to form a soluble complex which can be detected electrophoretically at pH 8.6. The protein composition of two different samples of corn varied, as one of the major protein components could not be detected in one sample.

In purification of seed proteins, removal of phytate ions is desirable. A method for their removal from protein using highly cross-linked anion exchange resin is described.

The extracts have enzymes capable of hydrolyzing the endogenous proteins. They have optimum activity at a pH of about 4 and are inactive at pH 7 or above at low temperatures.

The presence of water-soluble proteins in corn was first recognized by Chittenden and Osbourne (2). Study of saline extracts of corn exhaustively dialyzed against water indicated the presence of two albumins and some nonheat-coagulable protein referred to as a "proteose or peptone." The precipitate from dialysis of water and saline extracts was designated "globulin," although portions were redissolved only in dilute alkali.

Winterstein and Wünsche (20) observed water-soluble protein in corn which was not heat-coagulable. Work by Zeleny (22) led him to believe that the precipitate forming during dialysis of water extracts

[1] Manuscript received August 1, 1957. A portion of this work was presented at the 129th meeting of the American Chemical Society, April 1956, Dallas, Texas.

[2] Northern Utilization Research and Development Division, Peoria, Illinois, one of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture.

against water was "globulin." However, he was not able to detect the "albumins" described by Chittenden and Osbourne. He also observed the stable water-soluble proteins which were referred to as proteoses. Foster *et al.* (8) have analyzed preparations of corn proteins, including those from water extracts, electrophoretically in the presence of detergents. From the entire corn as many as four components were observed.

In this laboratory an extensive study of the proteins of corn is in progress. This report covers observations made on the nitrogenous compounds of high molecular weight present in water extracts of corn.

Materials and Methods

Preparations of protein were made from two commercially grown samples of corn: corn A was Dyar 444 and corn B was Schwenk 13. The shelled corn (16–18% moisture) was stored at 40°F. The moisture content was reduced to 10% at room temperature before the kernels were ground to a fine meal in a laboratory hammermill to pass 1/16-in. perforations. A large portion of the lipid material was removed by covering the meal with petroleum ether at 5°C. for 1 hour. After the meal was dried on a Büchner funnel, corn A contained 10.9% moisture, 1.20% nitrogen, and 0.27% phosphorus, while corn B contained 8.4% moisture, 1.72% nitrogen, and 0.30% phosphorus.

Although water extraction of corn is not a completed process in 24 hours (8), complete extraction was deemed unnecessary for preliminary work. As others (12) have noted, a stirring procedure was almost as effective for extraction, as shaking and low temperatures did not retard extraction. To prepare the extractions, 100 g. of meal and 500 ml. of water were stirred for 1 hour at 1°–2°C.

Nitrogen was determined by a conventional micro-Kjeldahl procedure using mercuric oxide as a catalyst in the digestion. Inorganic orthophosphate was determined by the Fiske-Subbarow method (7). The phosphorus in other forms, such as phytate, was converted to orthophosphate by digestion in boiling concentrated sulfuric acid with hydrogen peroxide as described by Pons *et al.* (14). Crystalline sodium phytate was prepared as described by Posternak (15).

To determine the loss of nitrogen and phosphorus by acid precipitation, the pH of 15-ml. aliquots of water extract was adjusted by careful dropwise addition of 0.1*N* hydrochloric acid with continuous stirring. At selected pHs the mixtures were transferred to volumetric flasks and made to a volume of 25 ml., where the pH was measured. The mixtures were centrifuged at low speed, and nitrogen and phosphorus were determined on aliquots of the supernatant. Values of

nitrogen and phosphorus lost by precipitation are reported as the percent of the original nitrogen and phosphorus.

For dialysis experiments the solutions were placed in cellulose casings. The solutions against which they were dialyzed were stirred continuously at 0°C. No evidence of fermentations or bacterial growth were noted even on experiments of several days' duration.

For removal of phosphorus compounds Dowex[3]1(10X) was used. The resin was cycled twice through the hydroxide and chloride form before conversion to the acetate or carbonate form by washing with solutions of sodium acetate or carbonate until the eluate was free of chloride ions. Preliminary experiments were performed on small columns as described by Van Etten and Wiele (18). The amount of nitrogen and phosphorus not adsorbed on passage of 5 ml. through the resin column was determined. In batch operations 40-ml. aliquots of extract were shaken periodically by hand during contact with weighed amounts of moist resin for 1 or 6 hours at 5°C. Precipitates formed were removed by centrifugation, and analyses were run on the clear supernatants to determine loss of nitrogen and phosphorus.

For removal of phosphorus compounds from preparations of proteins, a satisfactory technique was to allow 250 ml. of water to stand in contact with 5 g. of moist Dowex 1 (10X) in the acetate form for 1 hour at 5°C. The extract was filtered through glass wool to remove the resin. The extract was treated a second time with 5 g. of resin. Complete solubility of the protein at pH 2.5 indicated adequate removal of phosphorus compounds.

Electrophoretic experiments with the Perkin-Elmer Model 38 in a 6-ml. analytical cell were performed in veronal buffer of pH 8.6 and ionic strength of 0.1 with a current of 14 ma. at a temperature of 1°C. Since protein concentrations were unknown, preparations were assayed at a protein nitrogen content of 1.6 mg. per ml. Mobilities (μ) measured in the descending arm of the Tiselius cell are averages of five or six experiments in units of cm/volt sec $\times 10^{-5}$. Patterns are photographs taken at 1-hour intervals.

Increases in trichloroacetic acid (TCA)-soluble nitrogen of an extract during incubation periods were considered to be caused by autolytic enzymatic hydrolysis of the proteins present. TCA (6% final concentration) was added to aliquots of extract and the precipitate was removed by centrifugation. The remaining TCA solutions were then assayed for nitrogen. The pH was controlled by mixing the extract with appropriate buffers. The increases of TCA-soluble nitrogen

[3] Mention of firm names or commercial products under a proprietary name or names of their manufacturer does not constitute endorsement of such firms or products by the U. S. Department of Agriculture.

are expressed as percentages of the total nitrogen in the original extract.

Wet precipitates or residues were washed with methanol, acetone, and ether and were air-dried before analysis. Moisture was determined by drying samples at 100°C. over phosphorus pentoxide. Nitrogen and phosphorus content are reported on a dry basis.

Results

Composition of Extracts. The water extracts contained about 0.4 mg. of nitrogen and 0.5 mg. of phosphorus per ml., which was as much as 16% of the nitrogen and 95% of the phosphorus of the whole meal. The pH of the extracts ranged from 6.0 to 6.3.

Exhaustive dialysis of the extracts against water showed that about 37% of the nitrogen was dialyzable or nonprotein nitrogen. The proportion of nitrogen soluble in 6% trichloroacetic acid was about 35%. The dry solid (2.8% nitrogen) obtained on lyophilization of the extracts contained a large amount of nonprotein material.

Stability of Extracts. The water extracts were unstable at 5°C. as a material of low nitrogen content (3.6%) slowly precipitated. In 12 hours as much as 10% of the nitrogen became insoluble. At room temperature a white flocculation formed which was mostly protein (13.6% nitrogen). Both of the above actions were repressed by the addition of sodium chloride, so that the solutions were stable for several days at 5°C. On addition of the salt the solutions became clearer and a minor portion (1–2%) of the nitrogen precipitated. To utilize this protective influence by ions, solutions were dialyzed against 0.1 to 0.2 molar sodium chloride solutions rather than water.

Further instability was noted in the absence of added salt, as only 90% of the nitrogen in the solid resulting from lyophilization of the extracts was soluble. An insoluble residue was formed during freezing and thawing. Successive steps of freezing and thawing produced more of the residue, but the amount lessened each time until finally the action ceased. As much as 18% of the total nitrogen became insoluble.

Proteolytic Activity. Enzymatic hydrolysis of the endogenous proteins was assayed in these extracts. Table I shows there was an increase of TCA-soluble nitrogen, indicating that hydrolysis of the proteins occurred in the acid pH range at 40°C. but that the activity was depressed at low temperatures. There was practically no hydrolysis at a pH above 7 at 40°C. Thus, in the preparation of proteins from these extracts long exposure to acid pHs were avoided if possible.

Fractionation of Proteins. In previous work (2, 20, 22) the corn proteins of saline or water extracts were fractionated into albumins

TABLE I

INCREASES IN TRICHLOROACETIC ACID (TCA)-SOLUBLE NITROGEN DURING INCUBATION OF WATER EXTRACTS OF CORN

pH	Temperature	TCA-Soluble Nitrogen		Increase of TCA-Soluble Nitrogen
		0 minutes	60 minutes	
	°C.	mg.	mg.	%
7.7	40	0.56	0.58	1.1
6.5	40	0.54	0.60	3.4
4.5	40	0.56	0.74	10.0
3.8	40	0.54	0.71	9.7
3.8	26	0.48	0.56	4.6
3.8	0	0.48	0.48	0.0

and globulins by dialysis *versus* water on the assumption that only the globulins precipitated in solutions of low ionic strength. Consequently, it was suggested (2) that the more soluble globulins were present in water extracts as a result of the solubilizing influence of the inorganic ions of the corn. In our work the precipitate (I), occurring on dialysis of water extracts against water, contained about 10.4% nitrogen and 1.0% phosphorus; thus it was not all "globulin." A small amount of I redissolved in 0.5*M* sodium chloride. Dialysis of the solution *versus* water gave a precipitate which contained 17.1% nitrogen. The portion of I which did not redissolve in saline contained 8.1% nitrogen and 0.8% phosphorus. In these experiments, about 24% of the nitrogen precipitated during dialysis against water.

In comparison to dialysis techniques, a more adequate fractionation of the proteins was obtained when the pH of the extracts was lowered. The gradual increase of precipitate as the pH decreased is reflected in Table II, where an increasing loss of nitrogen and phos-

TABLE II

PRECIPITATION OF PHOSPHORUS AND NITROGEN WITH DECREASING pH OF WATER EXTRACTS OF CORN MEAL AT 25°C.

Final pH	Loss by Precipitation	
	Nitrogen	Phosphorus
	%	%
5.2	24.5	4.8
4.1	40.3	7.5
3.2	47.3	10.5
2.2	42.8	16.7
1.7	40.9	42.0

phorus from solution is seen. The amount of protein precipitated at a particular pH was higher at lower temperatures.

The precipitate (II), formed at a pH of 3.5, was roughly 10% nitrogen and 3% phosphorus. This large amount of phosphorus was not removed by washing with acetate buffer (pH 3.5) and evidently was

not an occluded impurity. Since the amount of both nitrogen and phosphorus depended on the exact pH, it was difficult to obtain reproducible preparations of the precipitate from the viewpoint of elemental analysis. It was evident that 60–70% of the precipitate at pH 3.5 was protein.

A portion of II was soluble in dilute saline at pH 7. A flocculation similar to II occurred when the pH of the redissolved protein solution was lowered, but the precipitation occurred over a narrower pH range and was complete at pH 4.2. The material was more soluble than II, was more reproducible analytically, and was mostly protein (15.6% nitrogen); it also had a high proportion of phosphorus (2.9%).

A major portion of precipitate II did not redissolve in water or saline even when the pH was adjusted as high as 8 or 9. This fraction is designated residue I (R-I). When the pH of the original extract was lowered to 3.5 and then raised to 7 without removal of II, the insoluble R-I also developed. R-I was a gray-green, gelatinous substance which formed a rather stable colloidal suspension in water. Addition of sodium chloride caused the particles of R-I to aggregate. The coloration increased on exposure to air and formed a hard, horny mass when dried. It contained about 12.6% nitrogen and was low in phosphorus (0.1 to 0.3%). Qualitative tests with anthrone reagent (10) indicated the presence of considerable carbohydrate.

All of the protein was not precipitated at pH 3.5. The material soluble at the pH contained nitrogenous compounds of high molecular weight as well as of low molecular weight. Some of the compounds of high molecular weight precipitated on saturation of the solution with ammonium sulfate.

Electrophoretic Analysis. Electrophoretic assay of preparations of the water extracts of corn at pH 8.6 and μ of 0.1 showed the presence of at least eight components producing boundaries. Most of the preparations contained large amounts of nondialyzable or slowly dialyzable materials which are not proteins. These components do not migrate but form large boundaries (peak 7 in the figures) which remain at the position of the Δ and ϵ boundaries. It is probable that these materials are water-soluble polysaccharides (5, 11).

About 90% of the nitrogen of a lyophilized extract dissolved in veronal buffer to give a solution of pH 8. Dialysis of the solution *versus* the same buffer for 20 hours removed compounds of low molecular weight and this equilibrated at pH 8.6 for electrophoretic assay. A typical pattern obtained from such a preparation after 180 minutes is shown in Fig. 1, A. Dialysis of the same preparation for an additional 40 hours provided patterns similar to B in Fig. 1. The change in

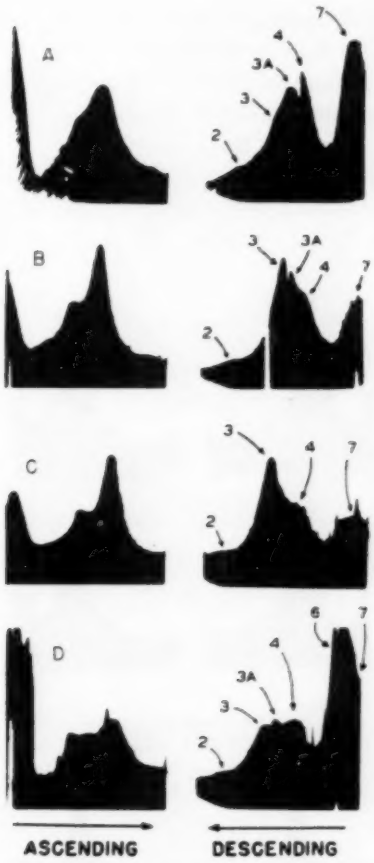

Fig. 1. Electrophoretic patterns of lyophilized water extracts of corn in veronal buffer of pH 8.6 and ionic strength of 0.1. All patterns are photographs at 180-minute intervals. A, 18-hour dialysis, corn B; B, 58-hour dialysis, corn B; C, 75-hour dialysis, corn B; D, 18-hour dialysis, corn A.

the patterns was accentuated by dialysis for a longer period as in Fig. 1, C. The proportion of the three components 3 (μ 4.3), 3A (μ 3.6), and 4 (μ 2.8) changed considerably, as 3 increased while 3A decreased so that it was not detected in Fig. 1, C. If the protein solution from A in Fig. 1 was allowed to stand at 0°C. without dialysis for 40–70 hours before a second assay, the pattern remained the same. Thus the change in the patterns resulted from dialysis and was not due to aging. The polysaccharides of stationary peak 7 are removed slowly during dialysis, as Fig. 1 shows, where the amount of 7 decreased in B and C.

Differences in the patterns of preparations made from the water extracts of two corn samples were noted. The preparations assayed in

A, B, and C in Fig. 1 are made from corn B. A similar preparation whose source was corn A gave the pattern of Fig. 1, D. The very slow-moving major component 6 (μ 0.5) of D in Fig. 1 is missing in A, B, and C. This component occurred consistently in preparations from corn A but was not extracted from corn B.

As previously described, a portion of the protein precipitated at pH 3.5 at 25°C. (11) redissolved at higher pHs. The resulting solution gave the pattern in Fig. 2, A, after 120 minutes. Two major compo-

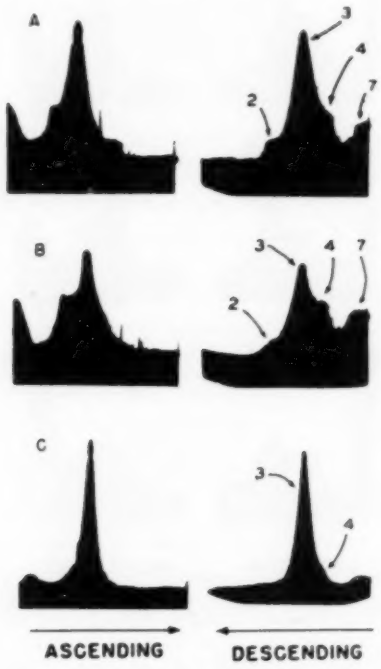

Fig. 2. Electrophoretic patterns of fractions which precipitate from water extracts of corn when the pH is adjusted to 3.5. All patterns are photographs at 120-minute intervals. A, the material precipitating at pH 3.5 at 25°C., which redissolves above pH 7. B, the material precipitating at pH 3.5 at 0°C., which redissolves above pH 7. C, the material from A was further purified by reprecipitation twice.

nents, 3 and 4, and minor materials were present. The pattern resembled that obtained in Fig. 1, C. The slow-moving component 6 was not detected in protein precipitated in the acid pH range. If the precipitations were carried out at 0°, the proportion of component 4 increased (Fig. 2, B).

The material, which remained soluble at pH 3.5 at 25°C., contained considerable protein. After concentration it gave the pattern in

Fig. 3, A, when prepared from corn A. There were only two major components, 4 and 6, with only minor amounts of other migrating material. There was a large amount of immobile material (polysaccharide). If the source of the latter preparation was corn B, then the

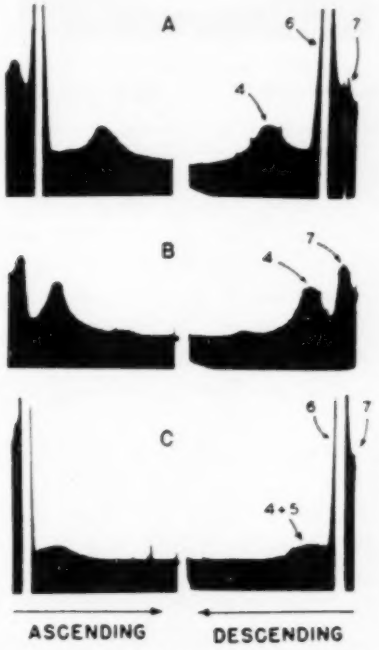

Fig. 3. Electrophoretic patterns of fractions which remain soluble when the pH is adjusted to pH 3.5. A, the material soluble at pH 3.5 at 25°C. from corn A. Time: 240 minutes. B, the material soluble at pH 3.5 at 25°C. from corn B. Time: 120 minutes. C, the material soluble at 3.5 at 0°C. from corn A. Time: 180 minutes.

very slow component 6 is missing from the pattern as in Fig. 3, B. If the precipitation at pH 3.5 was performed at 0°C., more protein was removed from solution. The pattern of the soluble protein from corn A then contains primarily the slow-moving component 6.

If the precipitate obtained at pH 3.5 (II) was reprecipitated twice, a material was obtained which was completely soluble at pH 7. This purified fraction gave an almost homogeneous pattern relatively free of polysaccharide (Fig. 2, C).

Lowering the pH of the water extract to 3.5 and then raising it to pH 7 produced the insoluble residue R-1. Assay of the remaining lyophilized solution gave the pattern of Fig. 4. There was a considerable decrease in the amount of component 3, suggesting it was specifically involved in the action of forming R-1.

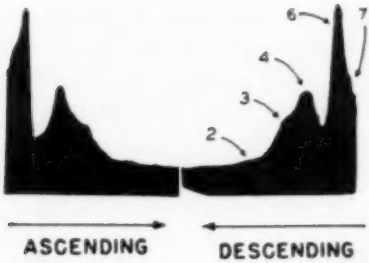

Fig. 4. Electrophoretic patterns of material remaining soluble after the pH of a water extract is lowered to 3.5 and then raised to 7.5. Time: 120 minutes.

In addition to the three major mobile components, three minor components were detected. The fastest moving or number 1 (μ 11.0) was not visible in any of the figures, but it was easily visible in patterns at 60 minutes. Component 2 (μ 7.2) was present in low concentration and usually appeared as a shoulder on the front edge of component 3. Component 5 (μ approximately 1.0) was not readily detected in most assays, but in a few experiments with purified fractions was observed definitely.

Removal of Phosphorus Compounds. About 70–90% of the phosphorus in corn has been reported to be present as phytin phosphorus (6, 14, 16, 19). In the water extracts used here, about 11% of the phosphorus was inorganic orthophosphate and 2% was from easily hydrolyzable phosphate esters.

Barré (1) has shown the need for removal of phytic acid in work with plant proteins. A similar requirement in purification of soybean proteins was shown by Smith and Rackis (17). Thus consideration was given to the phosphorus compounds and their removal, which was essentially the removal of phytic acid. Addition of calcium ions produced heavy flocculation which removed only a portion of the phosphorus (45–50%) and which had the undesirable aspect of precipitating some of the protein. Other divalent or trivalent ions gave similar results. The phosphorus compounds were not completely removed by dialysis against water. The procedure was slow, and the detrimental effects of aging have been noted above. Although almost 90% of the phosphorus was removed, an acid precipitate similar to II was obtained.

Partridge (13) has suggested the use of highly cross-linked anion exchange resins as molecular sieves. With such resins, small anions such as phytate penetrate the resin lattice to reach exchange sites. Larger molecules such as proteins, although they might exist as anions, would not adsorb extensively on the resin, as they could not penetrate the lattice. Extracts were percolated through columns of Dowex 1

(10X) to adsorb phytate, orthophosphate, amino acids, and other anions, while the proteins pass through into the effluent. Ninety-nine to one hundred per cent of the phosphorus was adsorbed on the resin column (chloride form), while only a portion of the nitrogen was removed from solution. Use of large columns was hindered by small amounts of precipitate forming which clogged the resin so that percolation became too slow.

In operations performed to determine the most effective conditions for phosphorus removal, Table III shows the batch process to be less

TABLE III

REMOVAL OF NITROGEN AND PHOSPHORUS FROM WATER EXTRACTS WITH ANION EXCHANGE RESIN IN ONE HOUR (BATCH PROCESS)

Form of Resin	Weight of Resin	Loss		Final pH
		Phosphorus	Nitrogen	
	g.	%	%	
a. Acetate	1.4	89	44	6
b. Acetate[a]	1.4	79	39	6
c. Acetate	0.7	58	36	6
d. Chloride	1.4	87	39	4
e. Carbonate	1.4	73	29	7
f. Acetate[b]	1.4	99	..	6

[a] Contact time was 6 hours.
[b] Three treatments with the resin.

efficient than column procedure. The acetate or chloride forms of the resin removed almost 90% of the phosphorus and about 40% of the nitrogen. Evidently the resin capacity was exceeded in experiment C. Since the final pH with the acetate form was higher and since phosphorus removal was effective in one hour, the acetate form was selected. Three treatments by the batch process removed 99% of the phosphorus.

The extracts treated with anion resin were water-clear and remained stable (with regard to solubility) for more than a week at 5°C. The dry material (3.0% nitrogen), obtained from lyophilizing resin-treated extracts, was completely soluble.

The precipitation of protein in acid pHs was not obtained in the extracts treated with anion exchange resin to remove phosphorus compounds. If the solutions were concentrated, a precipitation was observed at pH 5.3. The protein was all redissolved at pH 3.8. After the addition of phytic acid to these resin-treated extracts, acid precipitation of protein again occurred.

Discussion

By standard classical definitions the material precipitating from water extracts of corn on dialysis against water is designated globulin

(2, 20, 22). A native globulin is recovered from the precipitate, but it is only a portion of the total. The present results indicate that other actions cause precipitation during dialysis. First, there is the slow precipitation that occurs on standing at low temperature, which may be due to an aggregation similar to that described by Wolf and Briggs (21) in soybean proteins. Second, there is a removal of inorganic ions that exert a stabilizing influence which is apparent at room temperature. The precipitate occurring on dialysis is not an adequate measure of "total globulin" in water extracts. These factors make it difficult to effectively measure the globulins and water-soluble proteins by solubility criteria.

Although the water extracts contain heat-coagulable materials, we do not have conclusive evidence for the presence or absence of albumins. For the present, the nondialyzable nitrogenous compounds are considered as proteins and no attempts are made to classify or measure fractions based on classical definitions. Unpublished results from this laboratory indicate that water-soluble proteins exist in corn and are extracted with alkaline buffers of pH 9 but not with water. Also, similar proteins exist in corn which are soluble in saline solutions of very low ionic strength but which are only extracted from seeds with solutions of high ionic strength. Thus the ability of some proteins to be extracted does not reflect their solubility.

It has been recognized with various proteins (3) that an ionic reaction forms insoluble complexes between proteins and phytate ions. Since large amounts of phytate are present in water extracts of corn, the high phosphorus content of acid-precipitated protein described here indicates the same action occurs with these proteins. The results in Table II are similar to those reported by Courtois and Barré (4) with almond proteins. The percentages of phosphorus in the proteins precipitated in acid pH ranges are also similar.

When the phytate is removed from the water extracts, as with the anion exchange resin, a precipitation does not occur as the pH is lowered. If phytic acid is added to the phosphorus-free solutions, the acid precipitation (Table II) can then be obtained. This indicates the precipitation is due to formation of insoluble protein-phytate complexes. The electrophoretic evidence indicates that either component 6 does not form complexes with phytate or else the complexes are not insoluble under the conditions of our experiments.

Courtois (3) has pointed out the desirability of removing phytate from preparations of seed proteins. Methods previously described for removal of phytate (3) have been inadequate. The method described here removes phytate efficiently and rapidly while being a mild treat-

ment of the proteins. The use of anion exchange resins might be applied generally to purification work with seed proteins.

If the phosphorus-free solutions are concentrated to one-third the original volume, a portion of the protein precipitates in the range from pH 3.8 to pH 4.5. This precipitation is considered to be an isoelectric precipitation instead of the phytate complex precipitate of the original extract. Present work in this laboratory concerns the electrophoretic analysis and chromatographic separations of the proteins in the phosphorus-free solutions.

The work of others indicates that soluble protein-phytate complexes can exist (16, 19). To explain the electrophoretic assays described here, component 3A of the patterns may be considered as a complex which dissociates slowly as a dialyzable component is removed from the system. Component 3 is the protein moiety of that complex. Thus in Fig. 1 as the time of dialysis increases, the proportion of protein component 3 increases while that of the complex 3A decreases. Under the conditions studied, evidence that the other proteins are involved has not been established. This does not exclude complexing under other conditions. Attempts to re-form the complex of the dialyzable components with protein have been unsuccessful. However, in the light of other work it seems probable that the complex is between protein component 3 and phytate ions. Study of these reactions will be undertaken after further purification and separation of individual proteins.

One of the objectives of this work is to show possible genetic differences in the proportion of the individual proteins in corn. Such variations may exist, as protein component 6 was obtained from corn A but not from corn B. As our knowledge of the history of the corn sample was incomplete, it is possible that the protein was not extracted as a result of some other factor. The complete absence of the component in water extracts suggests its absence in the corn as a result of genetic difference. Further experimentation is in progress to confirm this observation.

The presence of phytate has aided in the fractionation of the water-extracted proteins. The original extracts are dilute with respect to protein, so that an acid precipitation does not occur in the absence of phytate. With the phytate present two of the three major components, 3 and 4, are precipitated as protein-phytate complexes. The complex of component 4 is more soluble at pH 3.5, so that precipitation at 25°C. gives a fraction enriched with 3. Further reprecipitation gives a fairly homogeneous protein under the conditions of assay. Component 4 is then obtained in enriched fraction by precipitation as a phytate

complex at the same pH at 0°C. A simpler preparation involves use of corn B which does not contain 6. Removal of 3 at pH 3.5 at 25°C. leaves a solution enriched in 4. The third major component, 6, is prepared in a highly purified form by removal of 3 and 4 at pH 3.5 at 0°C.

The electrophoretic evidence of Fig. 4 indicates that protein 3 leads to residue R-1, which is the insoluble portion of the precipitate at pH 3.5. The insoluble material is similar to that described by Hellot and Macheboeuf (9) with peanut proteins. They noted that the insoluble residue continues to form on reprecipitation of the acid-precipitated protein, until a "*fraction definitivement soluble*" was obtained. This corresponds to the purified fraction of corn protein in Fig. 2, B. The change in the characteristics of the protein which gives rise to R-1 depends on the presence of phytate, as Hellot and Macheboeuf have stated, but the low phosphorus content indicates the phytate is not retained in R-1. The carbohydrates of seeds must also be involved in the reactions.

The pH value at which the phytate-protein complex occurs and the effect of increasing ionic strength on that pH have not, to our knowledge, been reported. The ready crystallization of primary amines as amine hydrochlorides in an excess of hydrochloric acid may be explained as a repression of the ionization of the amino group by common ion effect. The solubility which depends on the ionization of the amino group is thus decreased. Similarly, a high concentration of ions would repress the ionization of amino groups of proteins. Reduction in solubility is not evident, since the other functional groups contribute to the solubility. Thus, with the decreased ionization, the reaction between amino groups and the phytate anions does not occur until a lower pH is reached.

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THE EFFECT ON FLOUR COLOR OF CLADOSPORIUM GROWTH ON WHEAT[1]

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ABSTRACT

As a result of cold, wet weather during harvest, much of the 1956 crop of wheat in England was superficially invaded by fungi with dark-colored mycelium, particularly *Cladosporium*. This led to poor color in flour milled from the wheat, and greatly limited the amount of native wheat suitable for commercial milling.

The dark-colored mycelium was present superficially on the beard end of the seed and, in severely affected samples, mycelium and sclerotia were abundant between the epidermis and the cross layer. Strips of epidermis were removed, washed to remove superficial contaminants, and cultured; most of these yielded *Cladosporium*. Flour milled from this wheat contained dark specks consisting of fragments of mycelium or sclerotia, fungus spores, or particles of pericarp carrying dark mycelium. Milling methods were investigated by which contamination of flour by minute dark specks, due to shattering of bran tissues, might be limited or avoided.

English wheat of the 1956 crop has been found characteristically to produce flour of poor color. The effect has been traced mainly to the growth of dark-colored fungi, especially *Cladosporium*, upon the grain, as a result of a delayed harvest due to exceptionally wet, cold harvest weather. Molds of this type have been previously described in connection with grain but not, to our knowledge, as factors affecting flour quality, and it seems worth while to put the main facts on record.

The presence of fungal mycelium on and in the pericarp of sound wheat is well known, the most commonly found types being *Aspergillus, Penicillium*, and *Alternaria* (6, 10). On occasions, usually associated with very wet harvests, the growth within the pericarp is sufficiently heavy to cause discoloration and spoilage of the grain. This has been described in most wheat-growing regions and variously named: black-point (U.S.A., 2, 5); black-point (India, 12); schwärze, Germany (1, 8); mouchetage (France, 9); puntatura (Italy, 11). As the names largely indicate, the discoloration affects principally one end of the kernel, on some occasions the germ, and on others the beard end. One or another of the dark-colored Demetiaceae has been recognized as being mainly responsible, *Alternaria, Helminthosporium*, and *Cladosporium* predominating on different occasions. There are indi-

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cations that, when the kernel discoloration is caused by *Helminthosporium* and sometimes by *Alternaria*, it is associated with a pathogenic infection of the whole plant and nonviability of the seed, but *Cladosporium* is reported to affect only the kernel and not to cause loss of viability. The investigations cited have been concerned with agricultural aspects of the fungal attack such as the effect on the grain as seed, and no reports have been noted which discussed its effect on the milling quality of the grain.

Materials and Methods

Wheat Samples. Some 40 different samples of English wheat were examined. The wheats had been grown in widely separated localities representative of the wheat-growing area of England.

Flour color grade values were determined by means of the Kent-Jones and Martin Color Grader (7).

Ash was determined on a 5-g. sample incinerated overnight at 600°C. in a silica basin.

Wheat Treatment and Milling Tests. Dry cleaning of wheat was carried out on the laboratory sieve separator and aspirator, and scouring on a Wolf laboratory scourer fitted with an indented metal casing. Washing was effected by immersion of the wheat, with agitation, for 60 seconds, followed by centrifuging. Milling was carried out by means of the laboratory MIAG mill, using break rolls with 18 corrugations per inch, smooth reduction rolls, and mechanically shaken sieves. Different milling systems were used at different stages of the investigation. The short system (A), used for testing variously treated grain, consisted merely of passing the wheat once only through the break rolls which were set sufficiently closely to produce a relatively heavy yield of break flour (12–17%) without unduly shattering the bran. The grind was sifted on a No. 12 standard silk, the throughs being used for the color test.

In system B, used in the purification study, the wheat was put through the first three breaks of the normal laboratory milling system. The combined semolina (sizings or coarse middlings) from these breaks, which amounted to 40% of the mill feed, was then closely graded, and the various grades were fed successively to the laboratory "purifier." This is simply a form of "gravity purifier," in which a horizontal air current is blown through a falling curtain of the stock; the more branny the material the further it is blown by the air. The machine is unsuitable for grading (making size separation), as in commercial purifiers which incorporate sieves. It does, however, make satisfactory separations of bran from endosperm, provided it is fed

with stock that has previously been sufficiently closely graded in particle size.

For the tests on various grades of flour prepared from different wheats, system C was applied. Appropriate stocks from the breaks, performed as in system B, were reduced so as to give three different grades of flour totaling about 70% of the wheat. Of these grades, the patent flour was made by reducing the first- and second-quality purifier throughs, such as those shown in Table II, from all the graded semolinas. The trend of separation of the fugus-contaminated bran was broadly the same in all grades as in that for the semolina through 32, over 40 GG, shown in Table II. The middle-grade flour comprised the first, second, and third break flours (totaling about 20% of the wheat), plus the flours obtained by reducing 1) the tails from the milling of the patent flour; 2) the "throughs of the tail sheets" from all semolina purifications; and 3) the break middlings. The low-grade flour was obtained by reducing the coarse and fine tails from the milling of the middle grade. No fourth break stocks were included in the preparation of any of these grades, the bran being left in the condition in which it was scalped from the third break grind. The bran amounted to 20% by weight of the initial wheat.

Identification of Fungal Types. The cultural technique described by Christensen (3) was used on small strips of epicarp mounted on Czapek agar in Van Tieghem cells. Preliminary surface-sterilization of the grain with mercuric chloride as used by Hyde and Galleymore (6) for sound grains was not successful. The grains were so severely weathered and affected by the mold that the epidermis was loose and cracked in many places. The solution penetrated to the mycelium on the inner surface of the epidermis, and no growth at all was obtained from strips so treated.

Christensen's method of preparing the grains was to wash in water for several hours, adding detergent, and shaking vigorously at intervals. As he points out, this method is again not applicable to grains with broken pericarp, as spores are carried through the breaks and cannot be washed out again.

The method finally adopted was to remove the selected strips of epidermis from untreated grains and wash the strips, after removal, according to the method which Christensen had applied to whole grains. This was apparently successful; three or four mycelia usually developed from each strip, but by constant observation most of them could be related to their point of origin in the subepidermal mycelium. Fruiting bodies were eventually produced from many of the mycelia. No attempt was made to recognize species.

Results and Discussion

General Extent of Effect on Flour Color. Flour of 70–72% extraction, milled from English wheat of the 1956 crop, has characteristically shown a color grade value of over 4, the normal value expected being about 2. The effect was roughly proportional to the amount of this wheat included in a mixed grist, thus:

Proportion of 1956 crop English wheat in grist (%)	0	25	50	75
Color grade value of flour	2	3	4	5

Furthermore, the effect on flour color of slightly decreasing flour extraction rate, or of fining-up the dressing covers by one or two numbers, has been surprisingly limited.

Appearance of Grain. While to the naked eye some of the wheat samples examined appeared reasonably bright, vitreous, and hard, as in the top and bottom rows of Fig. 1, under medium magnification

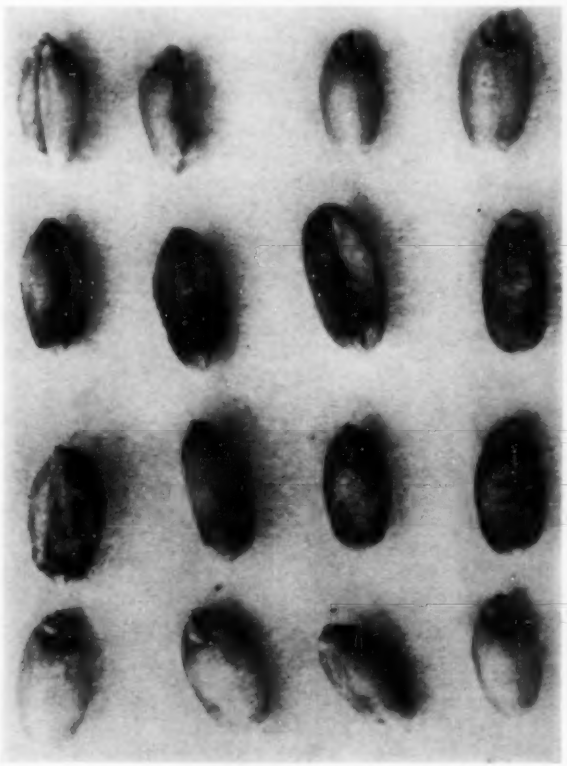

Fig. 1. English wheat grains of the 1956 crop showing different degrees of discoloration (×3). *Middle two rows:* typical grains from badly fungus-affected samples. *Top and bottom rows:* grains (for comparison) from other samples of normal appearance to naked eye.

many of the grains in these samples were observed to carry superficially some dark fungal growth in the neighborhood of the beard, as in Fig. 2. With the most obviously affected samples, such as those shown in the middle two rows of Fig. 1, the dark staining was most pronounced at the beard end, spreading to varying extents towards the germ. In these cases there was heavy development of the dark fungus beneath the epidermis, usually between this and the cross-layer. Figure 3 shows a magnified surface view of grain in a region where fungal hyphae were situated beneath the epidermis, and Figs. 4A and 4B show corresponding microscopical preparations. Figure 4A shows hyphae on the inner surface of the epidermis and Fig. 4B, proliferation of hyphae in the early stage of sclerotium development.

Many grains had a number of black particles or blisters, only the largest of which could be detected by the unaided eye. Grain, which to the naked eye appeared unweathered, sometimes carried two or three of these particles which were either round or took the form of streaks. Figure 5 shows a magnified surface view of both forms. Each particle is a specialized development of the fungus, termed a sclerotium, which serves as a resting stage. Figure 6 shows a large sclerotium,

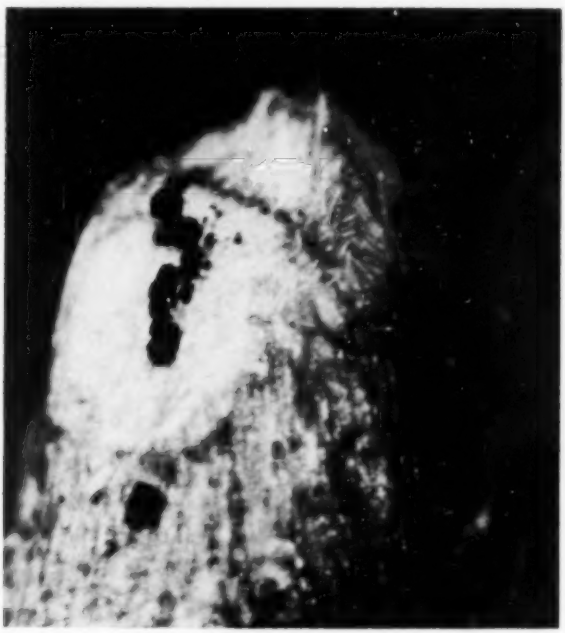

Fig. 2. Fungal growth on the surface of a wheat grain of the 1956 English crop (×30).

Fig. 3. View of the surface of a wheat grain of the 1956 English crop. The part of the surface selected is where fungal hyphae are situated beneath the epidermis (×30).

on the edge of the crease of a grain, which lay beneath the epidermis and has been partly uncovered by means of a dissecting needle. The sclerotia were found frequently to lie beneath the epidermis, though the largest might break through the surface of the grain.

Identification of the Fungus. From one or two samples no fungi could be persuaded to grow. This might possibly have resulted from excessive temperatures during farm-drying of the grain; sufficient heat would of course kill the fungus. Growth was obtained from several samples, however, and the following different types have been distinguished: *Cladosporium*, *Cephalosporium*, *Stemphyllium*, *Penicillium*, *Sporotrichum*, *Botrytis*, and *Alternaria*. Of these by far the most important was *Cladosporium*. It was responsible for the grain discoloration in the great majority of cases examined, producing a

coarse dark-colored mycelium and associated minute black sclerotia under the epidermis. On Czapek medium the mycelium produced had the "rosenkranz" form described by Demmler (4).

Normally, *Alternaria* is present in very small amount in almost every sample of sound grain (6, 10). It would therefore be expected to become predominant, given suitable conditions. On the other hand, *Cladosporium* is the "black mold" which ordinarily develops in the withering glumes of the ear during wet weather. In the present case it appears that initially the grain was normal. Later, with the ex-

Fig. 4A. Microscopical preparation corresponding to Fig. 3, showing hyphae on inner surface of epidermis (×500).

Fig. 4B. A microscopical preparation similar to that of Fig. 4A, but of another region, showing hyphal proliferation in early stage of sclerotium development (×500).

tremely wet conditions, *Cladosporium* transferred its activity from the glumes to the exposed beard end of the grain.

Nature of Effect on Flour Color. Examination under low-power magnification of flour samples, milled from affected wheat, invariably showed the presence of numerous dark specks. Many specimens of these were transferred by means of a needle point to wet mounts and microscopically examined. They were found typically to consist either of particles of beeswing (pericarp) carrying the dark fungus, of fragments

of detached mycelium, or of individual spores. Portions of the black outer layer of the sclerotia were also found, suggesting that to some extent the pustules are broken open during milling and fine fragments of dark mycelium become entrained in the flour. Many of the specks were smaller than the average flour particle. This would explain the fact that little or no improvement could be obtained through fining up dressing covers.

Improvement of Flour Color through Wheat Treatment. A collection of dirt in the beard, as in the crease, sometimes occurs in normal samples, but in good-quality wheats the beard is substantially free from any such debris. In the present samples some grains carried at their beard end spores and fragments of mycelium which were often lodged between the hairs. In other grains superficial fungal matter was clearly associated with chaffy material (including fragments of straw or glumes) which was adhering to the surface of the grain, evidently as a result of threshing the crop in a wet condition. Such chaffy material usually included groups of fungal spores as well as heavily infected tissue; the spores were repeatedly found in the affected flour

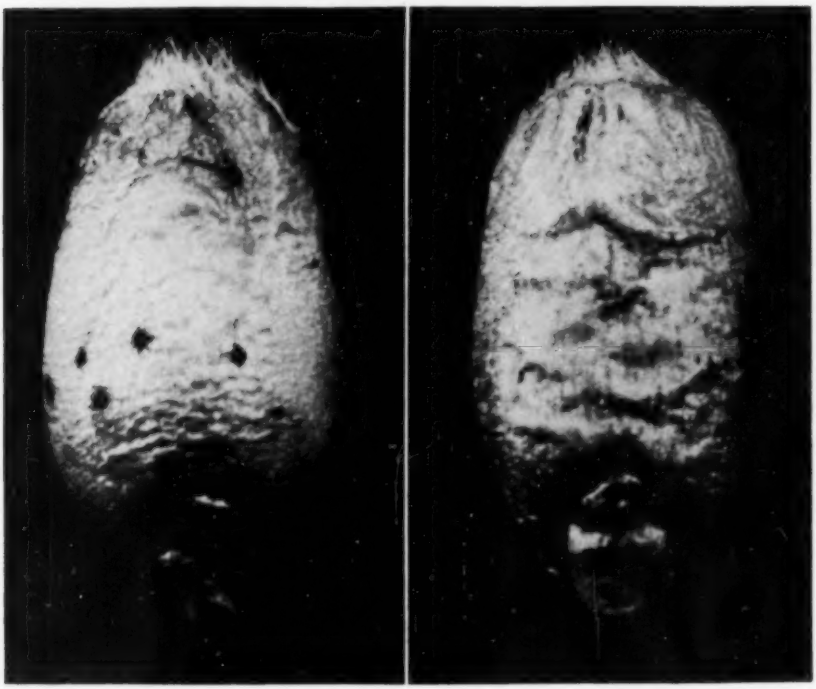

Fig. 5. Wheat grains showing magnified surface view of round (left) and streak (right) forms of sclerotia.

Fig. 6. Large sclerotium (indicated by arrow) on edge of crease of grain. Partly uncovered by means of dissecting needle (×40).

and semolina, but were not related to the fungus which was found to have grown beneath the epidermis.

Clearly, the superficial contamination should be removable by scouring or washing. This possibility was examined by means of the laboratory tests shown in Table I, carried out on a blend of English wheat samples. The last column in Table I shows color grade values determined on the flours prepared by means of laboratory milling procedure A (described under Materials and Methods). The value for sample No. 2, the English wheat after normal laboratory sieving and aspiration treatment, was 7.6. Exceptionally heavy aspiration, as in test No. 8, reduced this to 5.6, a considerable proportion of the discolored grains having been removed with the liftings, but this involved the rejection of a prohibitive proportion (17%) of the wheat. The heavy scouring treatment represented by test No. 5 removed only an

TABLE I

EFFECT OF VARIOUS PRELIMINARY TREATMENTS OF WHEAT ON FLOUR COLOR[a]

TEST NO.	DESCRIPTION OF WHEAT AS LABORATORY-MILLED	FLOUR COLOR[b]
1	Manitoba wheat (control)	2.3
2	English, sieved and aspirated in laboratory[c]	7.6
3	As 2 but also scoured once	6.4
4	As 2 but also scoured twice	6.2
5	As 2 but also scoured six times	4.9
6	As 2 but also washed and then scoured three times	5.5
7	As 2 but also scoured three times and then washed	3.9
8	As 2 but aspirated very heavily	5.6

[a] Flour was prepared by means of laboratory milling system A.
[b] Expressed in Kent-Jones & Martin Color Grader Units. The higher the reading, the poorer the color.
[c] The sieving and aspiration removed about 2% screenings.

additional 0.3% of light material and markedly improved the flour color. Washing following more moderate scouring treatment, as in test No. 7, gave the best improvement, but scouring the damp grain (test No. 6) was much less effective. During this test it was found difficult to ensure the escape of the damp discolored beeswing; under these conditions, the contaminant may have become redeposited to a considerable extent on the washed grain.

However, even the best improvement (test No. 7) following the wheat treatments shown in Table I resulted in a flour color inferior to that from sound wheat (test No. 1). This is understandable since, in the case of grains in which the fungal development is subepidermal, scouring or washing cannot be fully effective. With the sclerotial form, particularly, discolored material is so firmly attached and so well protected that the possibility of its removal through external abrasion is restricted. The action of fluted rolls, however, particularly when scraping closely as in the later breaks, or of low-grade reduction rolls, will shatter the fungal attachment quite extensively. As a result, the flour becomes contaminated with a large number of dark specks, many of which are extremely fine. This consideration pointed to the desirability of using the purifier system to divert to offal as much as possible of the fungus-affected bran instead of allowing it to remain in the feeds to the low-grade reductions.

Improvement through Purifier Adjustments. The possibility of this step depended on the finding that pieces of bran which are extensively discolored by fungus tend to be relatively light in weight (and therefore readily aspirated), because the fungal development has caused the outer layers of the bran to become partly lifted from the grain. In this state, these layers often tend to be peeled away by the fluted rolls; the peelings carry with them the fungal attachment, or some of it. Thus Table II shows results of an experiment with the laboratory purifier

(described, under milling system B, in Materials and Methods) on semolina, which had been graded between grit gauzes Nos. 32 and 40, and amounted to 11% of the parent wheat. Table II indicates that the

TABLE II

CHARACTER OF LABORATORY PURIFIER SEPARATIONS FROM SEMOLINA (SIZINGS OR COARSE MIDDLINGS) FROM FUNGUS-AFFECTED WHEAT

	SEPARATIONS IN ORDER OF INCREASING "LIGHTNESS"				
	1ST	2ND	3RD	4TH	5TH
Equivalent commercial description	First-quality throughs	Second-quality throughs	Throughs of tail sheet	Tails	Tins
Proportion (%)[a]	63.0[b]	24.0[c]	10.5[d]	1.5[e]	1.0[f]
Fungus contamination	....	Slight	More than second	Heavy	Very heavy

[a] As percentage of weight of feed to purifier.
[b] Endosperm particles, mainly bran-free but some with adherent bran.
[c] Flatter endosperm particles, bran-free; some germ; also considerable proportion of endosperm particles with adherent bran.
[d] About 50% bran; some scutellum; some detached beards.
[e] Mainly bran, with attached endosperm; considerable proportion of detached beards.
[f] Beards and beeswing.

proportion of pieces affected by fungus increased markedly on approaching the tail end of the purifier. The trend was found to be broadly the same in all grades as in that for the semolina through No. 32, over No. 40 GG, shown in the table. These results gave the general indication that part of the accepted reduction in extraction, when milling severely weathered grain, could be brought about to best effect by increasing the draft on purifiers, together with appropriate steps to ensure that as much as possible of the light bran extracted from the stock was by-passed to offal. This step would help the quality of the flours from the later parts of the reduction system and reduce the extent to which "finish" needed to be sacrificed there.

Contamination of the Endosperm and the Character of the Patent Flour. In none of the grains examined was the fungus found to penetrate the testa and gain access to the endosperm, but development of the grain in general was poor and irregular patches of dark-colored endosperm were frequently present. It appeared, however, that these patches were dark only because they had remained vitreous in consistency instead of becoming mealy and white as in well-developed grain. Imperfectly developed grains of normal samples had the same appearance and, when powdered, the vitreous endosperm in both appeared to be of good color. So far as could be seen from this inspection of the grain, the fungus had no direct effect on the endosperm, which differed from normal only in being not fully developed – the result most probably of the season rather than the fungus.

An alternative approach to this question was to examine the extent to which the color of flour reduced from relatively pure semolina was below the normal expected in patent flour of the grade in question. Such relatively pure semolina is exemplified by the first quality throughs shown in Table II – from which the fungus-affected bran is substantially absent even though the stock has been milled from affected wheat.

Accordingly, milling system C (Materials and Methods) was applied on the one hand to a sample of the fungus-affected 1956 wheat (from which the specimens described in Table II were prepared), and, on the other hand, to a sample of 1955 crop English wheat. This was a sample made up of equal parts of English-grown Atle (hard wheat) and Heines VII (soft wheat), both of which were in good condition after storage in the laboratory at about 13% moisture content. The mixture was damped to 15.5% moisture content before milling.

The results, including percentage yields, color grade values, and ash contents for the three grades of flour from each wheat sample are shown in Table III.

TABLE III

COLOR AND ASH CONTENT IN VARIOUS GRADES OF FLOUR LABORATORY MILLED FROM ENGLISH WHEATS OF THE 1956 AND THE 1955 HARVESTS

Flour Grade	1956 Crop			1955 Crop		
	Yield	Color Grade Values	Ash	Yield	Color Grade Values	Ash
	%		%	%		%
Patent	14.5	4.2	0.41	16.5	1.2	0.35
Middle	42.2	5.5	0.45	39.9	3.7	0.47
Bottom	13.6	9.3	0.67	14.3	5.7	0.61
Total	70.3	6.7	0.485	70.7	4.1	0.47

For the total flour and the flour of middle grade, Table III shows that while the ash contents are not dissimilar in the 1955 and 1956 samples, the color grade values are very different. The poorer color in the 1956 samples therefore appears to be due to a special discoloring factor, not to additional contamination with bran. With the patent flours, however, the 1956 sample appears to be affected both by additional bran contamination and also by the special discoloring factor; the presence of the latter factor follows from comparison of the 1956 patent sample with the 1955 total flour (here the 1956 sample contains less ash and therefore less bran, yet it is slightly poorer in color). Incidentally, it is anomalous that, while the ash content of the 1956 patent flour was distinctly higher than that of the 1955 (0.41 com-

pared with 0.35), the ash contents of the middle grades and of the total flours were fairly similar for the two years. It appears that in the milling of the fungus-affected wheat, for some unexplained reason, additional bran entered into the patent flour but not into the middle grade.

To avoid possible confusion, it should be mentioned that weighted means, calculated from the color grade values shown in the upper three rows of Table III, do not agree with the corresponding values, shown in the bottom row, which have been determined on the total flour. In the case of the ash contents, on the other hand, there is close agreement. This difference should be taken to mean, not that the accuracy of the color grade values is less than that of the ash determinations, but that the color grade values are not additive. This is evidently because the arbitrary scale of values is not related in a linear manner to the intensity of discoloration in the sample. Experience has shown, for example, that with a blend of high- and low- grade flours the determined value is higher by about 0.7 than the arithmetical mean of the values for the constituents.

Occurrence of Dark Fungal Specks on Bran-Free Semolina. Table III indicates that the patent flour from the 1956 wheat was affected by the special discoloring factor, in spite of the fact that the semolina from which it had been prepared contained very little bran that was contaminated with fungus. Close inspection of the purified semolina under magnification showed, however, that many of the "pure" semolina particles carried adherent dark minute specks. Microscopical examination of these specks showed that the majority were in fact fragments of fungal growth, not of bran. In Fig. 7 are illustrated a fragment of mycelium and alternaria spores removed from purified semolina. As the fungus sometimes occurs in the form of matted hyphae, it is readily understandable how such particles can become hooked mechanically to irregularities on the surface of an endosperm particle. Evidently, when the ground material is handled after leaving the break rolls, some specks transfer themselves from the bran to some of the pieces of endosperm. In keeping with this, examination under low-power magnification of the patent flour from the 1956 crop wheat showed the presence of dark specks. On transference by needle point to a wet mount, these were identified microscopically as fragments of fungal growth.

Investigation showed that the extent of the transference of fungal specks from bran to endosperm pieces was influenced by the nature of the scalping treatment; i.e., the manner in which, after a breaking operation, the coarse bran pieces are separated from the finer part of

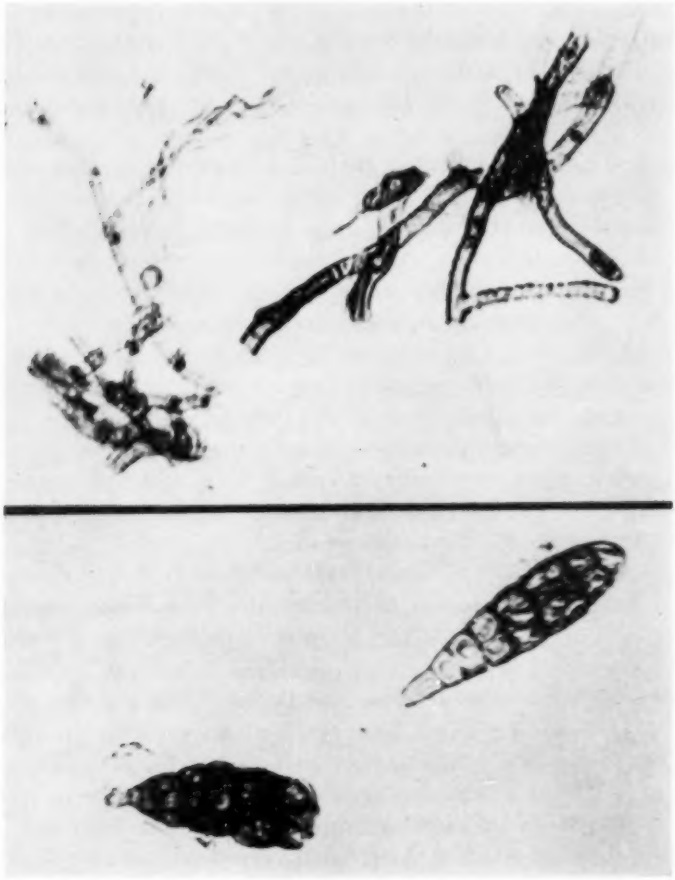

Fig. 7. Microscopical view of small dark specks removed from purified semolina: Upper, fragment of mycelium (×250); lower, *Alternaria* spores (×500).

the grind. Very gentle scalping by means of hand-operated sieves was found to decrease the color grade value of a patent flour from 1956 crop wheat by more than one unit, while the ash content was only 0.01% lower. Nevertheless, the color – at about three units – was still poor for a flour of this grade. The use of low-power magnification showed that, while the concentration of dark specks in the flour had been much reduced, it had not been eliminated. Therefore, while the poor color of the 1956 patent flours shown in Table III was certainly due in part to a residuum of contamination with fungal specks detached from the bran, it is possible that an intrinsic inferiority in color of the endosperm also contributed.

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THE EFFECT OF TEMPERATURE AND AIR VELOCITY ON THE FREEZING AND DEFROSTING RATES OF SOME BAKERY PRODUCTS

I. Dinner Rolls and Cinnamon Rolls[1]

K. KULP[2] AND W. G. BECHTEL[2]

ABSTRACT

Cinnamon and dinner rolls were frozen at 0°, −10°, and −20°F. and defrosted at 75°, 95°, and 115°F. at air velocities of 0, 150, 500, and 1,000 linear feet per minute. The freezing curves of dinner rolls indicated an apparent freezing point at 14°–16°F. No well-defined freezing point was observed for cinnamon rolls. Within the range of experimental conditions the freezing rate of the unwrapped products was increased more effectively by forced air than by lowering the freezer temperature from 0° to −20°F. On wrapped products the freezer temperature was a factor of major importance.

The defrosting rate of both wrapped and unwrapped products was greatly increased by elevation of the defrosting temperature from 75° to 95°F. A further increase to 115°F. was less effective. Forced air circulation was more effective in increasing the defrosting rate at 75°F. than at the higher temperatures.

Freezing as a method of preservation of freshness of bakery products is being used extensively in commercial practice. A successful application appears to be largely determined by the rate of freezing and defrosting. Cathcart (5, 6) and recently Pence *et al.* (11, 12) investigated extensively the rates of freezing and defrosting of white bread. It was concluded that rapid freezing rates tended to minimize the firming changes because bread remained in the "critical staling range" (65° to 20°F.) for only a short time. Defrosting rates were found to be of less importance in this respect. Research on the freezing of cakes was reported by Pence (10), who found that freezing and defrosting conditions had less effect on firmness than in the case of bread.

The literature on small yeast-raised products is fragmentary and the freezing recommendations vary a great deal. Charles (7) indicated successful freezing of soft dinner rolls at −10°F., Fenton (8) at −20°F. in moving air, and Beattie *et al.* (4) at −25°F. Industrial freezing of dinner rolls was described by Arnold (2). Sweet goods and coffeecakes were frozen commercially by Gordon (9) at a freezer temperature of 8° to 12°F. with relative humidity approximately 45%. Rapid defrosting

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[2] American Institute of Baking.

at elevated temperatures was indicated to be important, and the tempering of the products at 100° to 150°F. was found helpful in eliminating condensation. Tyor (14) obtained best results with sweet goods where the products were frozen at −10°F. and subsequently stored at 10°F. For single-temperature operation during freezing and storage, 10°F. was found sufficiently low.

This study was undertaken to obtain information on the effect of temperature, air velocity, and typical packaging on freezing and defrosting rates of dinner rolls and cinnamon rolls. The effect of these conditions on acceptability of the products will be reported separately. The investigated freezer temperature range was from −20° to 0°F., and the range of defrosting temperatures from 75° to 115°F. The air velocities in the freezer and defroster were 0, 150, 500, and 1000 ft. per minute. All these conditions can be readily met in current commercial practice.

Materials and Methods

Preparation of Rolls. The dinner rolls and cinnamon rolls were prepared at the experimental bakery of the American Institute of Baking according to the formulas given below. They were selected to give products of medium-rich composition. The dinner roll unit on which measurements were made consisted of nine rolls. The size of this unit was 7 by 7 by 1½ in. and its average baked weight was 8 oz. The cinnamon roll unit consisted of 12 rolls. It measured 8 by 8 by 1¾ in. and weighed 16 oz. The moisture content as determined by the vacuum oven method (1) was 33% for freshly baked dinner rolls and 26% for cinnamon rolls. (Table I.)

TABLE I
FORMULAS OF DINNER ROLLS AND CINNAMON ROLLS

	Dinner Rolls	Cinnamon Rolls
	% of flour	*% of flour*
Sponge		
Flour	60	76
Water	36	52
Yeast	3	8
Dough		
Flour	40	24
Water	29	none
Salt	2	2.5
Sugar	10	16
Nonfat dry milk	6	6
Shortening	10	16
Eggs	none	16

Freezing and Defrosting. The freezing experiments were conducted with freshly baked products equilibrated at 70°F. The experimental

freezer was equipped with an automatic control which kept the freezer within ±2°F. of the required temperature. To obtain the desired air-movement a section of the freezer was enclosed on four sides. At the center of this compartment a grilled tray was introduced to support the bakery products. An electric fan was placed at the back of the compartment. The speed and the distance of the fan were adjusted to give an average horizontal flow (across the product) of 150, 500, or 1,000 linear ft. per minute. The air circulation was measured in the empty compartment by a pin-wheel anemometer.

Defrosting measurements were made on products which were equilibrated at 0°F., for at least 16 hours. The defrosting cabinet was held automatically within ±2°F. of the required temperature. The air movement was produced in the same fashion as in the freezer. The products were supported on grilled trays. The humidity of the air of the defrosting cabinet was not controlled, but it was measured. The relative humidity of the air in the empty cabinet, determined by a wet-and-dry-bulb thermometer, varied from 40 to 60% at 75°F., from 30 to 40% at 95°F., and from 20 to 30% at 115°F.

Temperature of the rolls was determined at 5- to 10-minute intervals with 30-gage copper-constantan thermocouples which were inserted laterally into the geometrical center of the units and attached to a portable Brown potentiometer. The reported results are averages of at least two duplicate experiments. Freezing measurements were made in the temperature range from 70°F. to a temperature 10°F. above that of the freezer. For comparison of the principal variables, the time required to cool the products from 70° to 10°F., a range common to all three freezer temperatures, was used. The time required to produce this temperature change is referred to throughout this paper as the "cooling time." The "defrosting time" is defined as the time necessary for the temperature at the center of the product to increase from 0°F. to 70°F., and comparisons are made on this basis.

Packaging. Dinner rolls and cinnamon rolls were tightly wrapped in MST (moisture-proof, heat-sealing, transparent) cellophane of 300 gage thickness and heat-sealed. In addition to this, another typical packaging was used for cinnamon rolls. The rolls were packaged in a laminated paperboard tray of 0.018-in. thickness lined with a good quality greaseproof sheet, overwrapped with 300 MST cellophane and heat-sealed.

Results and Discussion

Effect of Freezer Temperature on Unwrapped Products. The effect of temperature on the freezing rate of unwrapped dinner rolls and cinnamon rolls is shown in Figs. 1 and 2. These results were obtained

without forced air circulation. The rate of heat transfer under this condition depends mainly on the temperature differential between the product and the cooling medium. Heat is removed from the unit by conduction and carried away by natural convection currents.

The freezing curves of dinner rolls in Fig. 1 indicate an apparent freezing point at 14° to 16°F., as is evident from the horizontal por-

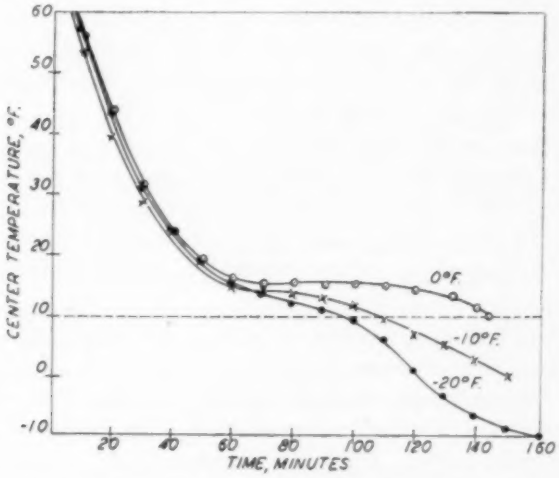

Fig. 1. Effect of temperature on the freezing of unwrapped dinner rolls.

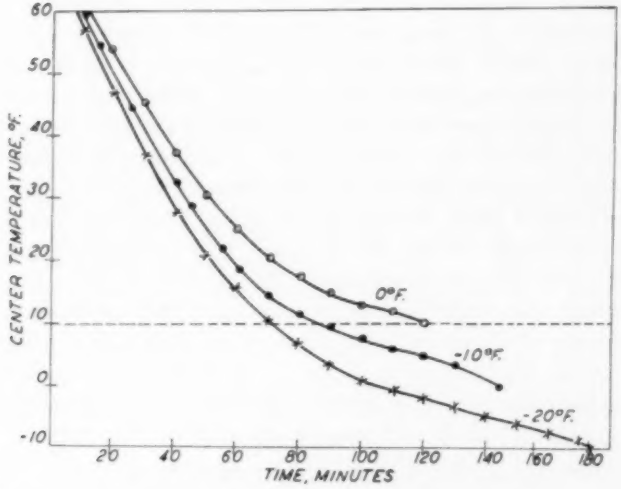

Fig. 2. Effect of temperature on the freezing of unwrapped cinnamon rolls.

tion of the freezing curve at 0°F. freezer temperature. This indicates that the major portion of ice is formed within this temperature range. Final solidification can hardly be expected at this point, since neither dinner rolls nor cinnamon rolls fulfill the requirements for isothermal freezing point which are met by pure substances and eutectic mixtures only. This has been shown by calorimetric studies on a series of food products by Short (13) and postulated from thermodynamic considerations by Bartlett (3). It is likely, therefore, that the products contained a certain amount of unfrozen liquid phase which remained above its eutectic point. The amount of this phase is, under isobaric conditions, generally a function of composition and temperature and is independent of the chilling rate.

The position of the apparent freezing point was generally somewhat lower at higher freezing rates. This is shown in Fig. 1 where the rate was increased by lowering the freezing temperature, and in Figs. 3 and 4 where this increase was effected by using forced air. Similar results were obtained by Pence (11) in his studies of the freezing of white bread.

Decrease of freezer temperature increased the freezing rate significantly. By comparison of the cooling time from 70° to 10°F. at different freezer temperatures, it was found that dinner rolls required approximately 29% less time to reach 10°F. with the freezer at −10°F. than with freezer at 0°F. This time decreased only an additional 9% when the freezer temperature was −20°F.

In the freezing curves of unwrapped cinnamon rolls of Fig. 2 no freezing point could be detected at 0°F. freezer temperature. There were slight breaks in the range of 5° to 10°F. at freezer temperatures of −10° and −20°F. which were more apparent from the individual results than from the plotted data. Cooling of the product throughout the entire temperature range was probably so rapid that the arrest of temperature due to the phase change could not be recorded more clearly. The moisture content of cinnamon rolls (26%) was approximately 7% lower than that of dinner rolls. The sugar concentration in the liquid phase of baked cinnamon rolls, disregarding fermentation losses, was 34% in contrast to 17% in dinner rolls. The content of added salt was 3.4% in dinner rolls and 5.3% in cinnamon rolls. The structure of cinnamon rolls was tighter than that of dinner rolls. Because of the lower amount of air in the structure of cinnamon rolls its insulating effect was less than in the more porous dinner rolls. All of these conditions seem to augment the rate of heat transfer.

The lowering of the freezer temperature from 0° to −10°F. decreased the cooling time (70° to 10°F.) of unwrapped cinnamon rolls

by 27%, a reduction comparable to that for dinner rolls. An additional decrease of 13% was obtained at −20°F. These results were analogous to those obtained by Cathcart (5) and Pence (11) on white bread and indicate that the transfer of heat through the rolls is less of a rate-limiting factor than might be expected in view of their porous cellular structure.

Effect of Circulating Air on Unwrapped Products. Introduction of moving air into the freezer performs a twofold function. It increases the mass rate of flow which passes the heat-transfer surface and decreases the boundary film layer which surrounds the product. The effect of air velocity on the freezing rate of dinner rolls with freezer at 0°F. is illustrated in Fig. 3. The cooling time (70° to 10°F.) of unwrapped dinner rolls decreased with each air speed increment. A low air velocity, 150 ft. per minute, had only a slight effect (an 8% decrease). The intermediate velocity, 500 ft. per minute, reduced the cooling time by 29% and gave a rate equivalent to that at −10°F. in still air. The high velocity, 1,000 ft. per minute, reduced the cooling time by a total of 44%. Heat transfer under this condition proceeded at a significantly higher rate than at −20°F. in still air. At all three air velocities the actual freezing zones were readily apparent, although they were gradually depressed and of shorter duration as the ambient air velocity increased.

The effect of air velocity on freezing unwrapped dinner rolls with freezer at −20°F. is shown in Fig. 4. Although the low air velocity shortened the time of freezing significantly, the major increase in

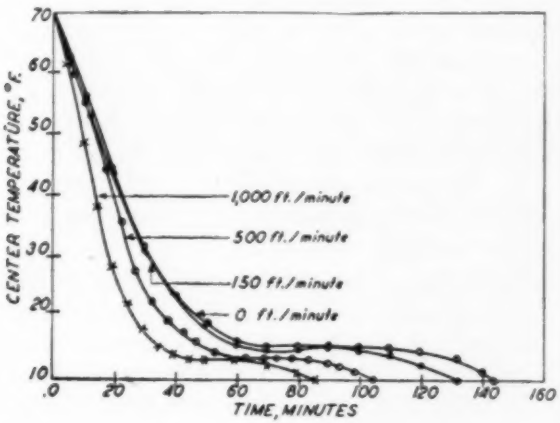

Fig. 3. Effect of air velocity on the freezing of unwrapped dinner rolls at 0°F. freezer temperature.

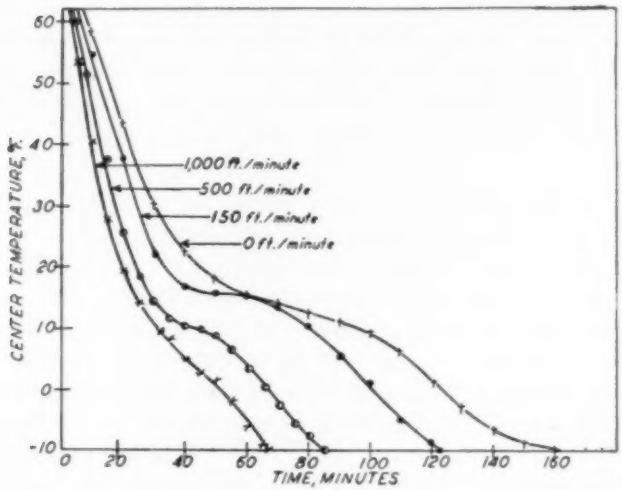

Fig. 4. Effect of air velocity on the freezing of unwrapped dinner rolls at −20°F. freezer temperature.

freezing rate was observed when the air velocity was raised to 500 ft. per minute. The time necessary for the product to pass through the freezing zone was reduced with each air increment. At the highest air speed, 1,000 ft. per minute, it became so short that it could not be recorded. The cooling time (70° to 10°F.) in still air was 100 minutes. This was reduced by 18% at 150 ft. per minute, by 55% at 500 ft. per minute, and by 66% at 1,000 ft. per minute air velocity.

The freezing rate of unwrapped cinnamon rolls was greatly increased by moving air. This is illustrated at 0°F. freezer temperature in Fig. 5. The cooling time (70° to 10°F.) in still air was 120 minutes. The major reduction of this time, a 42% decrease, was produced at an air velocity of 150 ft. per minute. Further increases of the air velocity to 500 and 1,000 ft. per minute reduced this time by 48% and 60%, respectively. Similar effects were observed at −20°F. (Fig. 6). The cooling time in still air was 69 minutes. It was shortened by 39% at 150 ft. per minute, by 42% at 500 ft. per minute, and by 49% at 1,000 ft. per minute air velocity.

Effect of Packaging. The effect of packaging on the freezing of dinner rolls and cinnamon rolls is shown in Figs. 7 and 8, respectively. The solid lines indicate the effect of air velocities on the cooling time (70° to 10°F.) at 0° and −20°F. freezer temperatures. The broken lines were plotted in addition to these in the same figures to show the times necessary to reduce the center temperature of the products to −10°F. at the freezer temperature −20°F.

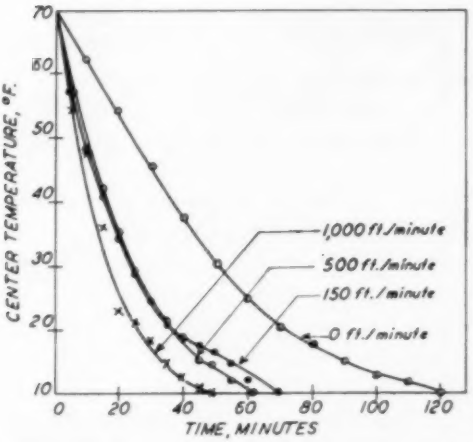

Fig. 5. Effect of air velocity on the freezing of unwrapped cinnamon rolls at 0°F. freezer temperature.

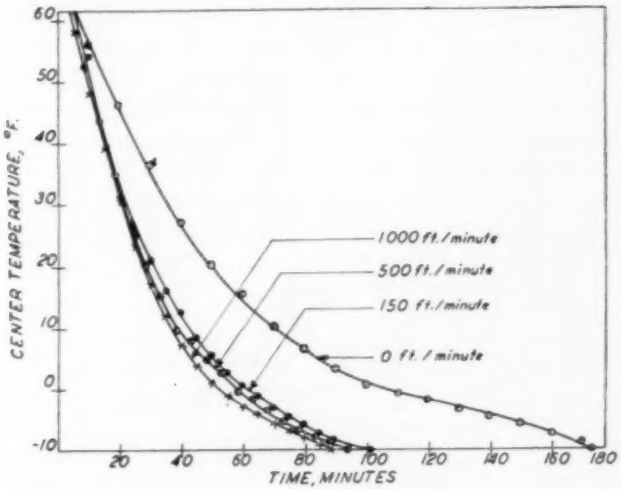

Fig. 6. Effect of air velocity on the freezing of unwrapped cinnamon rolls at −20°F. freezer temperature.

It is evident that forced air circulation had less effect in decreasing the cooling times of wrapped than unwrapped dinner rolls. This is especially apparent at 0°F. where the highest air velocity reduced the cooling time only to that of the unwrapped dinner rolls in still air. The cooling time (70° to 10°F.) of the wrapped dinner rolls was more effectively shortened by lowering the temperature from 0° to −20°F. than by using an air velocity of 1,000 ft. per minute at 0°F. At −20°F.

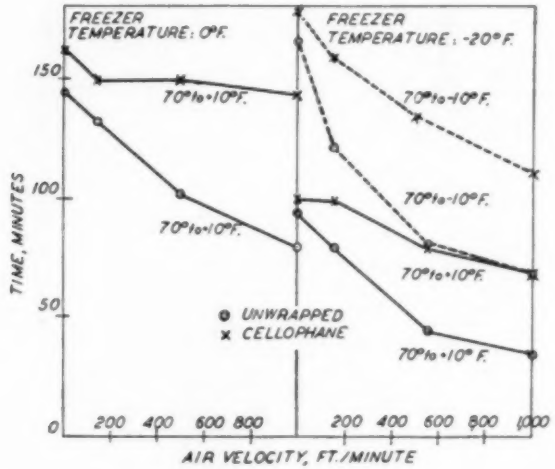

Fig. 7. Effect of packaging on the cooling times (70° to 10°F.) of dinner rolls at 0° and −20°F. freezer temperatures and air velocities from 0 to 1,000 linear ft. per minute.

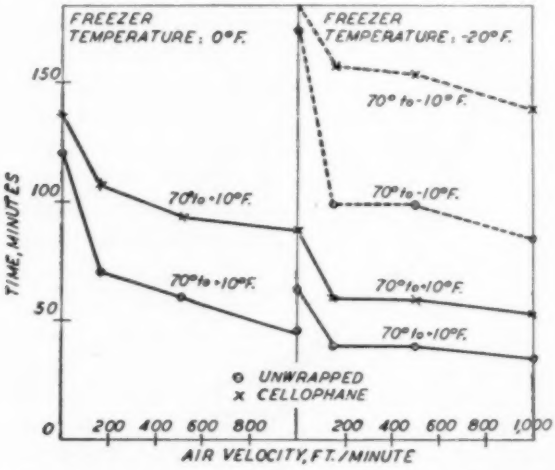

Fig. 8. Effect of packaging on the cooling times (70° to 10°F.) of cinnamon rolls at 0° and −20°F. freezer temperatures and air velocities from 0 to 1,000 linear linear ft. per minute.

in still air the wrapped dinner rolls cooled somewhat faster than the unwrapped dinner rolls at 0°F. with an air velocity of 500 ft. per minute. Forced air circulation was relatively more effective in reducing the cooling time (70° to 10°F.) of wrapped dinner rolls at −20°F. than at 0°F. At −20°F. and 1,000 ft. per minute the cooling time of

wrapped dinner rolls was somewhat less than that of the unwrapped items at 150 ft. per minute.

Cooling times of wrapped and unwrapped cinnamon rolls are given in Fig. 8. The effect of a freezer temperature of −20°F. without forced air on the cooling time of the cellophane-wrapped rolls was equivalent to that of 0°F. with an air velocity of 1,000 ft. per minute. At both temperatures the major decrease in cooling time of the wrapped rolls due to circulating air was produced with a velocity of 150 ft. per minute. Further increases of air flow decreased the cooling time only a small amount. Under conditions of maximum air flow (1,000 ft. per minute) at freezer temperatures of both 0° and −20°F., the cooling time of the wrapped products was materially longer than that of the unwrapped at an air flow of 150 ft. per minute.

The results obtained with cinnamon rolls packaged in a paperboard tray and cellophane overwrap were not plotted in Fig. 8. They showed that the paperboard tray increased the thermal resistance only slightly more than the plain cellophane wrapper. The cooling time under this condition was on the average 12 minutes longer at 0°F. and 4 minutes longer at −20°F. than that of cellophane-wrapped cinnamon rolls.

Defrosting. Typical defrosting curves of unwrapped dinner rolls and cinnamon rolls are shown in Figs. 9 and 10. Dinner rolls, which exhibited a definite freezing zone, passed through the melting zone rapidly, since this physical change took place at a high temperature differential. Defrosting of cinnamon rolls proceeded without any evi-

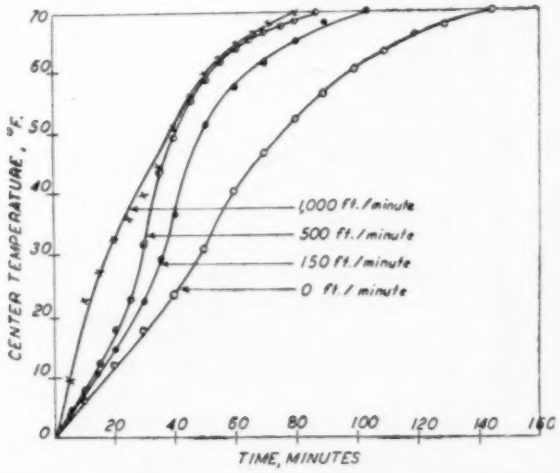

Fig. 9. Effect of air velocity on the defrosting of unwrapped dinner rolls at 75°F.

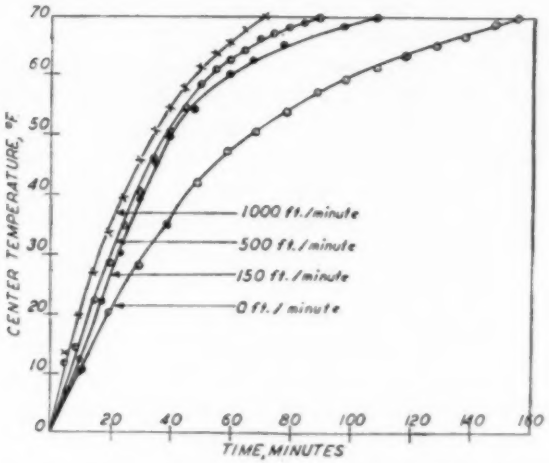

Fig. 10. Effect of air velocity on the defrosting of unwrapped cinnamon rolls at 75°F.

dence of melting point and the defrosting curves appeared to be mere reversions of the freezing curves. Similar results were obtained at 95° and 115°F.

The effects of temperature, air velocity, and packaging on the defrosting times of dinner rolls are clearly evident from Fig. 11. Increase of the defrosting temperature from 75° to 95°F. produced a

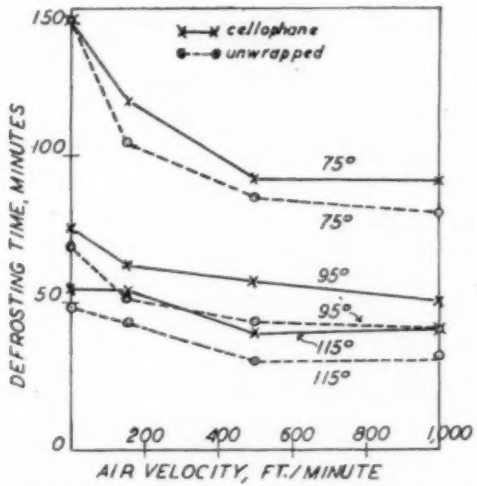

Fig. 11. Effect of defrosting temperatures on the defrosting times of unwrapped and wrapped dinner rolls at air velocities from 0 to 1,000 linear ft. per minute.

major reduction of defrosting time. The further increase of temperature to 115°F. was less effective. Whereas air velocities up to 500 ft. per minute produced material reductions of the defrosting times at all three temperatures, the increase of air velocity to 1,000 ft. per minute had a negligible additional effect. The reduction of defrosting time at 75°F. due to air velocities up to 1,000 ft. per minute was less than that caused by the increase of temperature from 75° to 95°F. At 95°F. forced air at 1,000 ft. per minute reduced the defrosting time of the rolls to that at 115°F. in still air.

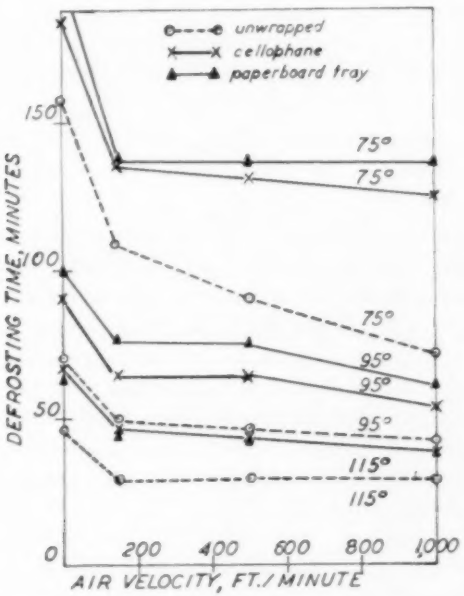

Fig. 12. Effect of defrosting temperature on the defrosting times of unwrapped and packaged cinnamon rolls at air velocities from 0 to 1,000 linear ft. per minute.

The cellophane wrapper retarded the defrosting time of dinner rolls in still air only slightly, but it became a significant barrier in moving air. This retarding effect was less pronounced on defrosting than on freezing. Within the range of air velocities from 150 to 1,000 ft. per minute the defrosting times of wrapped dinner rolls were increased 16% compared to the unwrapped, whereas the cooling times (70° to 10°F.) were extended by 38%.

The results plotted in Fig. 12 illustrate the effect of temperature, air velocity, and packaging on the defrosting time of cinnamon rolls. Whereas the increase of the defrosting temperature from 75° to 95°F.

was of major importance in reduction of the defrosting time, further increase to 115°F. was less effective. Significant reductions were gained at all three defrosting temperatures by employing a low air velocity (150 ft. per minute). There was no additional advantage in raising the air velocity to 500 or 1,000 ft. per minute except for defrosting unwrapped cinnamon rolls at 75°F., where material shortening of the defrosting time was observed with each air increment. At the highest flow of air the defrosting times of unwrapped cinnamon rolls at 75° and 95°F. were comparable to those in still air at 95° and 115°F., respectively. The defrosting time of wrapped products at 95°F. and 1,000 ft. per minute was comparable to that at 115° in still air. At 75°F. the highest speed of air was not sufficient to reduce the defrosting time to that observed at 95°F. in still air. The cellophane wrapper increased the defrosting time an average of 31% in still air and 45% in air circulating at rates from 150 to 1,000 ft. per minute. The corresponding increases for the paperboard tray with cellophane overwrap were 39% and 52%.

In these experiments dinner rolls behaved generally like white bread in the sense that the freezing curves showed a freezing plateau. High air velocity and low temperature were necessary to reduce the duration of the freezing zone. In contrast to dinner rolls, cinnamon rolls froze in a manner analogous to that of cakes. No distinct freezing zone was apparent and only low air velocities were needed to cause a major increase in the freezing rates. This indicates a definite connection between thermal behavior and composition which, when fully understood, could become the basis for rational prediction of freezing characteristics of bakery products.

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THE INHIBITION OF BETA-AMYLASE BY ASCORBIC ACID[1]

ARTHUR W. ROWE[2] AND C. EDWIN WEILL

ABSTRACT

The inhibition of beta-amylase by ascorbic acid has been found to be noncompetitive. This contrasts with the competitive type of inhibition caused by parachloromercuribenzoate. The nature of this inhibition and its reversal by very small amounts of cysteine suggest that the ascorbic acid is acting by some mechanism other than a direct reaction with the essential sulfhydryl groups of the enzyme.

The action of ascorbic acid on beta-amylase has been studied by several investigators (1, 2, 3, 4, 5, 6, 10). In one of the early papers Hanes (1) demonstrated that other enediols such as reductone and dihydroxymaleic acid also inhibited the action of the enzyme. In each case the activity of the enzyme returned when the enediol was oxidized.

Seshagirirao and Giri (10) showed that ascorbic acid caused a reversible inhibition, whereas ascorbic acid plus cupric ion caused an irreversible inactivation of beta-amylase. The extent of this irreversible reaction was far in excess of the inactivation caused by separate amounts of either ascorbic acid or cupric ion. These authors claim that the reaction in the presence of cupric ion was associated with the oxidation of ascorbic acid. The action of ascorbic acid alone was attributed to the influence of the enediol grouping on the enzyme.

Ito and Abe (2–6) proposed a mechanism to explain the irreversible inactivation of beta-amylase with ascorbic acid in the presence of cupric ions, but no experiments were conducted in the absence of cupric ions.

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[2] Submitted in partial fulfillment of requirements for the Master of Science degree.

The importance of free sulfhydryl groups in the activity of beta-amylase has been demonstrated (11), and it seemed plausible to expect a reaction between the ascorbic acid and the sulfhydryl group of the enzyme. The current study was conducted to determine whether such a reaction could be detected.

Materials and Methods

The beta-amylase was obtained from the Wallerstein Co. Inc. (New York, N.Y.). The enzyme, prepared from barley, was free of alpha-amylase activity and contained only inorganic salts as impurities.

The ascorbic acid was Lot 9636, obtained from Hoffmann-La Roche, Inc., Nutley, N.J.

The concentration of cysteine necessary to prevent inhibition of the enzyme by ascorbic acid was determined by measuring the activity of 2 mg. of enzyme at 25°C. on 100 ml. of 1% soluble starch containing the ascorbic acid, 0.05*M* acetate buffer at pH 4.5, and the cysteine.

The noncompetitive nature of the ascorbic acid inhibition was determined by adding the enzyme to the solution containing starch, 0.05*M* acetate buffer at pH 4.5, and ascorbic acid. Two levels of ascorbic acid concentration were used for at least three different starch concentrations. To prove conclusively that this was a reversible system, some of these values were redetermined by adding the ascorbic acid to the enzyme, and then adding an aliquot of this solution to the starch solution.

The activity of the enzyme in each case was calculated as mg. of maltose produced per mg. of enzyme acting on a solution of Takamine soluble starch (Takamine Laboratory, Inc., Clifton, N.J.) at 25°C. in the presence of 0.05*M* acetate buffer at pH 4.5. The maltose was determined by an iodine thiosulfate method (8). A 5-ml. aliquot of the reaction mixture was added to sodium carbonate solution to stop the enzyme action. Iodine was added to a measured portion of this solution. This was allowed to stand for 30 minutes at 20°C., acidified, and the excess iodine back-titrated with 0.005*N* thiosulfate.

When initial velocities were required, they were obtained by extrapolation and calculated as mg. of maltose per mg. of enzyme per minute.

The residual ascorbic acid concentrations were determined with the Beckmann Model D.U. Spectrophotometer at 265 $m\mu$ in the presence of 0.005*M* acetate buffer at pH 4.5. Ascorbic acid has been found to obey Beer's Law in concentrations from 1 to 5×10^{-5} molar. The aliquot of the reaction mixture was added to 1 ml. of 0.01*M* cysteine, and then diluted to 100 ml. This concentration of cysteine had been

found to be adequate to prevent further oxidation of the ascorbic acid, but not great enough to reduce the dehydroascorbic acid back to ascorbic acid.

The reaction mixtures were analyzed for cupric ion using the diethyldithiocarbamate test, and none was detected even though the reagent will reveal the presence of as little as one part in ten million.

Results

Table I shows the amount of cysteine necessary to prevent inhibition of beta-amylase by fairly large excesses of ascorbic acid.

TABLE I

CYSTEINE REVERSAL OF ASCORBIC ACID INHIBITION OF BETA-AMYLASE

Enzyme Preparation	Concentration of Ascorbic Acid	Concentration of Cysteine	Maltose / Enzyme[a]
mg	$\times 10^3$ *molar*	*molar*	*mg*
2	. .	. .	1.04
2	1	. .	0.19
2	1	1×10^{-5}	1.00
2	1	1×10^{-4}	0.38
2	5	. .	0.05
2	5	1×10^{-4}	1.01
2	5	1×10^{-5}	1.05
2	5	1×10^{-6}	0.18

[a] Represents mg. of maltose per mg. of enzyme produced in 30 minutes at 25°C.

The inhibition caused by a 5×10^{-3} molar concentration of ascorbic acid was completely eliminated by a cysteine concentration of 1×10^{-5} molar. This was a ratio of one part of cysteine to 500 parts of ascorbic acid. The ability of such a low concentration of cysteine to overcome the inhibition caused by a large excess of ascorbic acid suggested that the inhibition was not the result of a direct reaction between the enzyme and the ascorbic acid.

Table II shows the inhibition caused by two different concentrations of ascorbic acid on several concentrations of the starch substrate. Figure 1 is a plot of the values in Table II by the method of Lineweaver and Burke (7). Figure 1 demonstrates that the inhibition is noncompetitive.

Table III shows the effect of a typical competitive agent, parachloromercuribenzoate. Figure 2, a plot of the values in Table III by the method of Lineweaver and Burke (7), is a graph typical of a competitive inhibition. This is in contrast with the inhibition plotted in Fig. 1.

The ultraviolet absorption spectrum of ascorbic acid was exam-

TABLE II

ACTIVITY AND INHIBITION AT VARIOUS SUBSTRATE LEVELS

CONCENTRATION OF STARCH SOLUTION	CONCENTRATION OF ASCORBIC ACID SOLUTION	INITIAL VELOCITY [a]
%	$\times 10^4$ *molar*	
0.3	..	0.0267
0.4	..	0.0307
0.4	1	0.0205
0.4	2	0.0170
0.6	..	0.0340
0.6	1	0.0230
0.6	2	0.0187
1.0	..	0.0383
1.0	1	0.0263
1.0	2	0.0227
2.0	..	0.0400
2.0	1	0.0310

[a] Reported as mg. maltose per mg. enzyme per minute by extrapolation from data recorded at 15 and 30 minutes, using 2 mg. of enzyme preparation in 100 ml. of reaction mixture.

TABLE III

ACTIVITY AND INHIBITION AT VARIOUS SUBSTRATE LEVELS

CONCENTRATION OF STARCH SOLUTION	CONCENTRATION OF PCMB SOLUTION	INITIAL VELOCITY [a]
%	$\times 10^7$ *molar*	
0.4	2	0.0173
0.4	3	0.0123
0.6	2	0.0200
0.6	3	0.0160
1.0	2	0.0270
1.0	3	0.0217

[a] Reported as mg. maltose per mg. enzyme per minute at 25°C. by extrapolation from data recorded at 15 and 30 minutes, using 2 mg. of enzyme preparation in 100 ml. of reaction mixture.

ined to determine whether cysteine caused a shift in the usual peak at 265 mμ. Mole ratios of cysteine to ascorbic acid from 0.002 to 1000 were examined. There was no change in the location of the peak. When the cysteine concentration was about 1000 times the ascorbic acid concentration, there was a 2% drop in the value of the extinction coefficient at 265 mμ. This was not considered significant.

Discussion

Reversal of the inhibition of beta-amylase by ascorbic acid upon addition of cysteine has been considered indicative of a simple reaction between ascorbic acid and the sulfhydryl groups of the beta-

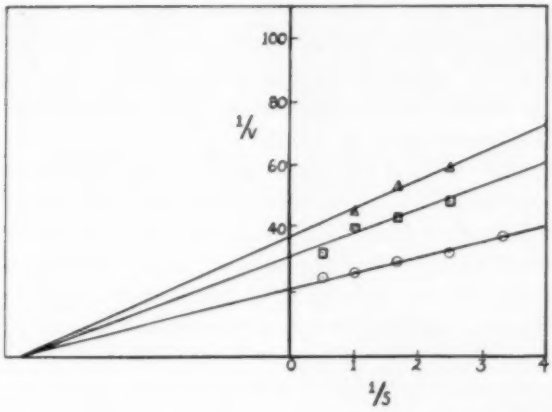

Fig. 1. Double reciprocal plot of ascorbic acid inhibition of beta-amylase. V, initial velocity; S, starch substrate concentration; ⊙, enzyme hydrolysis with no inhibitor; ⊡, hydrolysis with 1×10^{-4} *M* ascorbic acid; △, hydrolysis with 2×10^{-4} *M* ascorbic acid.

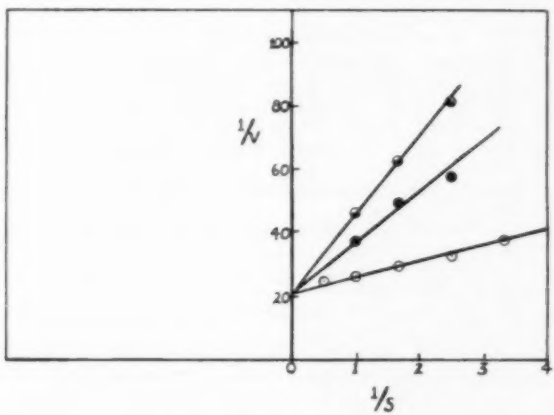

Fig. 2. Double reciprocal plot of parachloromercuribenzoate (PCMB) inhibition of beta-amylase. V, initial velocity; S, starch substrate concentration; ⊙, enzyme hydrolysis with no inhibitor; ●, hydrolysis with 2×10^{-7} *M* PCMB; ◒, hydrolysis with 3×10^{-7} *M* PCMB.

amylase. Similar relationships have been shown with other reagents for this enzyme (11) as well as for other enzymes with active sulfhydryl groups.

However, these experiments show that no simple stoichiometric relationship exists, as a very small amount of cysteine can reverse the inhibition caused by a large excess of ascorbic acid. If the inhibition were the result of a stoichiometric reaction, then there should be a marked loss of activity even in the presence of 10^{-5} molar cysteine.

The noncompetitive nature of the reaction also suggests the absence of a simple reaction of the inhibitor with the sulfhydryl groups in the enzyme. The effect of a direct action on the active grouping in the enzyme is noted in the competitive type reaction obtained with parachloromercuribenzoate in Fig. 2.

Neilands and Stumpf (9) state that "noncompetitive inhibitors apparently combine with an enzyme at a point other than the attachment of the substrate. They are then able to exert an effect on the active site even though they are situated some distance away." This may be the case of the ascorbic acid inhibition of the beta-amylase. While the vitamin may be affecting the sulfhydryl group, it is probably not by any direct reaction with it.

The absence of any change in the ultraviolet absorption spectrum of ascorbic acid in the presence of cysteine only shows that there is no reaction which can be detected by this type of measurement. While these results do not prove the absence of such a reaction, they do add to the evidence already accumulated.

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THE AEROBIC MICROBIOLOGICAL POPULATION OF PRE-FERMENTS AND THE USE OF SELECTED BACTERIA FOR FLAVOR PRODUCTION[1]

ROBERT J. ROBINSON, THOMAS H. LORD,
JOHN A. JOHNSON, AND BYRON S. MILLER

ABSTRACT

The total bacterial population of pre-ferments buffered with salt or with nonfat dry milk (according to the formula of the American Dry Milk Institute) tended to increase during the first 2 hours of fermentation and to decrease thereafter. The bacterial population in all except the salt-buffered pre-ferment decreased by 75–95% during 6 hours of fermentation. In salt-buffered pre-ferments the bacterial population remained relatively constant. Approximately 65 different aerobic microorganisms were identified in pre-ferments. Eighteen were identified in flour and 36 in hot- and cold-processed salt-rising-yeast cultures. Most bacteria belonged to the genera *Micrococcus, Aerobacter, Proteus, Bacillus,* and *Lactobacillus*. Bacteria in compressed yeast contributed most of the total bacterial population to pre-ferments. Few undesirable organisms (coliforms and micrococci) were found in pre-ferments after 6 hours of fermentation.

Acceptable flavors and odors were obtained in bread by the use of selected microorganisms. The best bread resulted from the use of *Lactobacillus bulgaricus* 09 or *L. bulgaricus* 09 plus a buttermilk culture. Members of the genus *Lactobacillus* were the predominant microorganisms in aged sponge-dough. Several microorganisms were lyophilized and used successfully as a dry mix in bread production.

The flavor of dough and bread is influenced by the action of microorganisms, some of which are more important than others. Yeast is the most important microorganism used in breadmaking, although Wardall (19) concluded from a study of 33 selected yeast cultures that flavor in bread was not determined by yeast alone. Baker and Mize (2), however, attributed the flavor of bread to the ingredients plus products developed by yeast, and products developed in the crust during baking. Other microorganisms, including some of those found in commercial sour cultures, are responsible for producing certain characteristic bread flavors and aromas.

The desirability of encouraging the growth of certain types of bacteria during bread fermentation has received little attention. Some organisms that produce the short-chain organic acids may enhance the flavor of the product. The presence of these acids in large quantity,

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however, would be undesirable. Certain microorganisms that produce specific organic products are desirable only in some special breads.

Fuller (6) divided the microorganisms associated with bread producton into three groups: The purified strains of the yeast, *Saccharomyces cerevisiae;* the desirable bacteria which aid in gluten development and produce certain flavor characteristics; and the undesirable microorganisms such as *Bacillus mesentericus (B. subtilis)*[2] torulae, and molds. Fuller (6), who was concerned with those microorganisms involved in the production of essential flavoring components, made a study of the microflora of "leaven." *Lactobacillus panis acidi*[3] was predominant in all samples, while *Streptococcus lactis* and others occurred in small numbers. He found, in experimental breadmaking, that *L. panis acidi,* in addition to yeast, produced bread with flavor and quality superior to that of bread in which yeast alone was added.

According to Schulz (18), sour dough must contain two types of microorganisms to yield successful bread. These microorganisms producing both lactic and acetic acid were identified as *Bacterium panis fermentati (Lactobacillus brevis)* and *Bacillus lactis acidi (Lactobacillus lactis),* respectively. He stated that the undesirable bacteria such as the coliforms and *B. mesentericus (B. subtilis)* are always encountered in flours and dough and produce spontaneous fermentation which may cause serious defects. The influence of these harmful bacteria could be reduced greatly or sometimes entirely by other aroma-producing microorganisms.

Jörgensen (9) and Heinemann and Hefferan (8) isolated bacteria having all the characteristics of *Bacillus bulgaricus (Lactobacillus bulgaricus)* from starters of salt-rising bread. These starter mixtures were composed of corn meal, sodium bicarbonate, milk, and salt. Kohman (11) compared bread made by the "Sauerteig" method with that made by the salt-rising method and found that gaseous fermentation in the latter is due entirely to bacteria, whereas the leavening power of the "Sauerteig" owes its origin primarily to yeasts.

Knowledge about the species of microorganisms making up the microflora of wheat flour was very limited prior to the investigation of Amos (1) and Kent-Jones and Amos (10). They isolated, from wheat flour, *Bacillus coli (Escherichia coli), B. perfringens (Clostridium perfringens), B. subtilis,* several strains of *B. mesentericus (B. subtilis),* and three unknown species which they designated No. 3, No. 4, and No. 11. They stated that the number of *B. coli (E. coli)* and *B. perfringens (C. perfringens)* was small in normal wheat flour but that the occur-

[2] The terminology for all bacteria is that used in Bergey's Manual (3). When an author has used an older name it appears first, followed by the Bergey name in parentheses.

[3] Species description not listed in reference 3.

rence of *B. subtilis* was common. Strains of *B. mesentericus (B. subtilis)* were present in most wheat flours. Microorganism No. 3 (a micrococcus) constituted the majority of the microorganisms present. Microorganism No. 4 (a coccobacillus) and No. 11 (a small bacillus) usually were present but in smaller numbers than No. 3. Of the microorganisms present, No. 3 was identical with *Micrococcus ureae-liquefaciens* Flügge (see footnote 3). Culture No. 4 appeared to be a member of the genus *Flavobacterium* and No. 11 appeared to belong to the genus *Achromobacter*.

The recent attention given pre-ferment and continuous breadmaking processes has pointed out the need for knowledge concerning the production of desirable flavors in pre-ferments. The purpose of this study was to isolate and identify the aerobic bacteria of pre-ferments, flour, and salt-rising cultures and to study their effect on bread flavor. No attempt was made to study the anaerobic bacteria or molds.

Materials and Methods

Pre-Ferment Formulas and Method of Preparation of Pre-Ferments. The pre-ferment formulas used in this study are listed in Table I. The yeast was thoroughly suspended in 100 ml. of sterile distilled water at

TABLE I

BASIC PRE-FERMENT FORMULAS

INGREDIENTS	KIND OF PRE-FERMENT			
	ADMI	SUGAR	FLOUR	SALT-BUFFERED[b]
	g	g	g	g
Water	320.0	320.0	320.0	320.0
Dextrose (or sucrose)	21.0	21.0	21.0	21.0
Yeast food[a,b]	3.5	3.5	3.5	...
Malted wheat flour	1.8	1.8	1.8	1.8
Sodium chloride	7.0	7.0	7.0	7.0
Nonfat dry milk	42.0	...	...	...
Compressed yeast	14.0	14.0	14.0	14.0
Flour	...	...	42.0	...
Brew improver[b]	...	...	...	2.1

[a] Arkady type.
[b] Standard Brands, Inc., New York 22, New York.

26°C. and combined with the dry ingredients. Additional sterile distilled water at 26°C. was used to rinse the vessel in which the yeast had been suspended. Each pre-ferment was placed in a sterile jar with a cover and incubated at 30° ± 0.1°C.

Preparation of Media. All media used in the study were prepared according to the Difco Manual (5) or to the manufacturer's directions. The general aerobic population was isolated and studied on nutrient

agar, starch agar, nutrient broth, gelatin agar, and litmus milk. Coliform bacteria were isolated and counted, using violet-red bile agar. *Lactobacillus* species were isolated and counted on tomato-juice agar and orange serum agar. Yeasts were isolated and counted on Sabouraud dextrose agar. Food-poisoning strains of *Micrococcus* species were isolated and counted on staphylococcus medium No. 110.

Determination of the Microbial Population of Pre-Ferments. One-milliliter samples of the pre-ferments were withdrawn after 0, 2, 4, 6, and 24 hours. These samples were plated in decimal series in each medium (5) at dilutions ranging to one one-billionth of the original strength. These plated samples were incubated at 37°C. and counted at the end of 48 and 72 hours to determine the number of total aerobic and facultative bacteria, yeast, lactobacilli, coliform bacteria, and staphylococci. Slides were made of each pre-ferment for visual determination of the types of microorganisms present.

Using the same pre-ferment mixtures without yeast, dilutions and platings were made and counted in the same manner as for the pre-ferment made with yeast in order to determine the bacterial population of the ingredients, thus excluding the yeasts.

Identification of Isolates. All bacteria isolated were tested on starch, milk, and gelatin media before baking tests were made. These aerobic and facultative organisms were first classified according to Gram reaction and morphology into the following classes: Gram-positive, spore-forming and nonspore-forming bacilli; Gram-negative bacilli; and Gram-positive cocci. For testing the biochemical reactions, media from those listed by Lord (12) were selected according to the type of organisms found.

Preparation of Cell Suspension for Inoculation of Pre-Ferments. A 10-ml. portion of nutrient broth was inoculated with a particular organism and incubated for 24 to 48 hours at 37°C. The culture was transferred into 1 liter of nutrient broth and incubated for 24 to 48 hours at 37°C. The broth culture was then centrifuged for 30 minutes and the cells washed twice with sterile, distilled water. The cells were resuspended in distilled water and the cell concentration determined.

Determination of Cell Concentration. The number of *Escherichia coli* L 145 cells per ml. for the inoculation of pre-ferments was determined, using a Skue Heper Nephelometer, model 61. The cell suspension was diluted in order that 1 ml. added to each pre-ferment would produce a final cell concentration of 1.25×10^5 per ml. of pre-ferment.

The number of cells of *Micrococcus pyogenes* var. *aureus* L 41 per ml. for the inoculation of pre-ferments was determined using a Petroff-

Hauser counting chamber. Another count was made using the Nephelometer for *Escherichia coli* L 145. The dilution of the organism was similar to that for *E. coli* L 145.

Preparation of Organisms for Use in the Baking Test. Each organism to be tested for flavor production was maintained as a stock culture in 10 ml. of sterile, reconstituted evaporated skimmed milk. A second 10-ml. portion of reconstituted evaporated skimmed milk was inoculated with these cultures and incubated for 24 hours at 37°C. This second 10-ml. inoculum was transferred to 100 ml. of sterile reconstituted evaporated skimmed milk, incubated at 37°C. for 72 hours, and used to replace 100 ml. of water in an American Dry Milk Institute (ADMI) pre-ferment mixture used in baking. The dough formula was adjusted for the amount of milk solids in the pre-ferment.

Judging Bread Flavor and Odor. Bread flavor and odor were examined using the triangle test (13). After tasting a preliminary sample of bread, each judge was given three samples, two of which were identical. Each participant was cautioned to proceed slowly, to take as nearly as possible the same amount of sample for each taste, to rinse the mouth with water between samples, and to pause after each taste in order to avoid interference between samples.

Results and Discussion

General Microbiology of Pre-Ferments. The total bacterial counts for the ADMI, sugar, flour, and salt-buffered pre-ferments increased

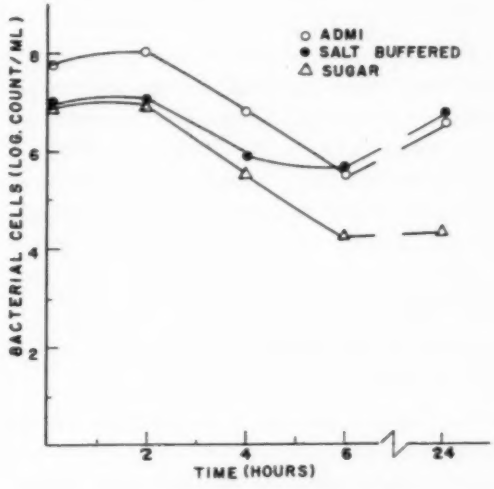

Fig. 1. Bacterial counts per ml. of pre-ferment made with yeast at 30°C.

slightly at 30°C. through the second hour (Fig. 1), declined between the second and the sixth hour, and increased again during the sixth to the twenty-fourth hour of fermentation. The results of counts on flour pre-ferment are not graphed because they parallel the other three sets of graphs. The early growth probably was due to the readily available food supply for the predominant number or groups of organisms present at the start of the fermentation. These organisms thrive on native protein, sugar, and salts, and make simpler food nutrients such as amino acids, peptides, and simple sugars available for the succeeding group of organisms. The drop in the bacterial count beginning after the second hour and continuing through the sixth hour of fermentation may be due to the first group of organisms' using up the foods available to them, or to the possible production of some inhibitory material which restricts growth or kills the other organisms present. The rise in the number of organisms from the sixth to the twenty-fourth hour may be due, in part, to the growth of new or resistant groups of organisms which were present in very small numbers at the start of fermentation. According to the identification of isolates at the end of 6 hours of fermentation, the majority of these organisms was of the genus *Lactobacillus* which is known to consist of "late starters." They usually grow best in the presence of sugars and after other less acid-tolerant organisms have died (7). This group of organisms can withstand high acid conditions and may be present in high numbers in doughs and certain dairy products. At temperatures higher than that studied (30°C.), the organisms grow faster and die off more rapidly. This has been pointed out by Prescott and Dunn (16).

Separate studies were made of the bacterial population contributed by freshly milled flour, Kohman's Salt-Rising-Yeast mixtures, and pre-ferment mixtures made without yeast. The pre-ferment mixtures containing no yeast demonstrated a low bacterial count (Fig. 2), but the freshly milled flour showed the lowest count of all the materials tested. The decline in bacterial population has been attributed to the production of alcohol during fermentation (4, 14), exhaustion of food supply, and/or lack of oxygen. Robinson *et al.* (17) (see page 306, this issue) did not confirm these conclusions.

The yeast counts remained relatively constant in all pre-ferments. These data corroborate Choi's (4) findings.

The compressed yeast used in this study was investigated to determine if it were the source of bacteria. The findings shown in Figs. 1 and 2 agreed with those of Choi (4) that most of the high bacterial count in the pre-ferment was due to compressed yeast. These organisms

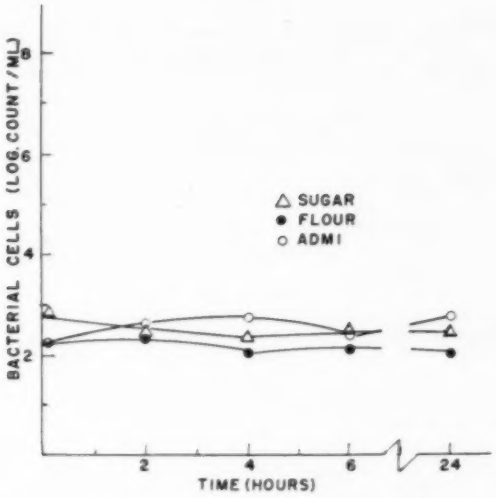

Fig. 2. Bacterial counts per ml. of pre-ferment made without yeast at 30°C.

might not be harmful to either the pre-ferment or the consumer and do not detract from the usefulness of yeast.

Identification of Selected Isolates. Microscopic observations of Gram-stained smears of all colonies found on the countable plates of all pre-ferments, freshly milled flour, and Kohman's Salt-Rising-Yeast mixtures (hot and cold) indicated the presence of Gram-positive, aerobic, spore-forming bacilli; Gram-positive coccus forms; Gram-negative bacilli; and Gram-positive, nonspore-forming bacilli. The majority of the Gram-negative bacilli fermented lactose readily with evolution of acid and gas. Observations of Gram-stained organisms selected from aged sponge dough indicated the presence of Gram-positive, nonspore-forming, short and long, thin bacilli. The isolates to be identified were keyed according to *Bergey's Manual of Determinative Bacteriology*, 6th edition (3), unless specified otherwise. The names and the percent occurrence of identified organisms appear in Table II.

Production of Bread Flavor in Pre-Ferments by Selected Microorganisms. The bacterial sources for flavor production in pre-ferments were colonies selected from countable plates of freshly milled flour, the four pre-ferment processes, Kohman's Salt-Rising culture, sponge dough, and certain pure cultures. Since protein and starch make up a large proportion of dough and bread, physiological reactions on milk and starch were performed to determine whether the isolates were proteolytic, saccharolytic, or relatively inert. When an organism was

TABLE II

PERCENTAGES OF SPECIES OF BACTERIA FOUND IN PRE-FERMENTS AND OTHER INGREDIENTS

ORGANISMS	PRE-FERMENT (ZERO HOUR)				KOHMAN'S SALT-RISING CULTURE		FRESHLY MILLED FLOUR
	ADMI	Salt-Buffered	Sugar	Flour	Hot	Cold	
Achromobacter butyri	4.5	..	..	..	..	..	..
A. liquefaciens	4.5	..	..	..	..	..	..
A. stationis	..	..	..	..	..	..	4.5
A. aerogenes	27.3	5.2	5.2	5.5	..	..	..
Bacillus brevis	4.5	4.5	..	..	..	..	4.5
B. coagulans	4.5	..	..	..	10.0	9.0	..
B. cereus var. *mycoides*	4.5	4.5	..	..	..	..	..
B. firmus	..	..	..	..	..	4.5	..
B. laterosporus	..	..	..	..	5.0	..	9.0
B. lentus	..	..	..	..	..	4.5	..
B. macerans	..	5.2	5.2	..	..	4.5	..
B. megatherium	..	..	..	..	..	4.5	..
B. polymyxa	..	..	..	5.5	..	..	..
B. pumilus	..	..	..	..	15.0	4.5	4.5
B. subtilis	22.8	..	..	..	50.0	18.2	18.2
Erwinia tracheophila	..	..	..	..	5.0	..	..
Lactobacillus brevis	..	..	..	..	..	4.5	..
L. buchneri	..	..	..	..	..	4.5	..
L. bulgaricus	..	5.2	5.2	..	..	..	..
L. casei	..	..	..	5.5	..	..	..
L. delbrückii	..	..	..	..	..	4.5	..
L. fermenti	..	..	..	33.4	..	..	..
L. helveticus	9.0	5.2	5.2	..	..	..	..
L. leichmannii	..	..	..	5.5	..	4.5	13.7
L. plantarum	4.5	5.5	5.5	..	..	..	..
Micrococcus aurantiacus	..	..	..	..	..	4.5	..
M. candidus	4.5	5.2	5.2	..	..	4.5	..
M. caseolyticus	..	..	..	..	5.0	4.5	4.5
M. epidermidis	..	..	..	..	..	..	9.0
M. flavus	4.5	4.5	..	..	..	..	..
M. ureae	..	12.8	15.8	..	..	..	4.5
M. varians	..	..	..	..	..	..	4.5
Proteus rettgeri	..	..	5.2	..	..	..	..
P. vulgaris	..	10.5	10.5	33.3	..	..	4.5
Pseudomonas sp.	4.5	4.5	5.2	..	..	..	..
Unknown bacteria and molds	0.4	27.2	31.8	11.3	10.0	18.8	18.6
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0

found to be highly proteolytic or highly saccharolytic, it was deleted from the baking tests.

The flavor and odor of bread and toast made with selected microorganisms from freshly ground flour, Kohman Salt-Rising culture, and ADMI, sugar, and flour pre-ferments were judged subjectively by several judges for acceptability. When bread and toast, containing specific microorganisms, were acceptable, baking trials were repeated. The final selections are recorded in Table III. Microorganisms that survived

TABLE III

RESULTS OF BAKING BREAD WITH SELECTED SINGLE STRAINS AND COMBINATIONS OF DESIRABLE MICROORGANISMS

MICROORGANISMS ADDED	ODOR EVALUATION	
Source or Treatment and Name	Fresh Bread	Toasted Bread
Standard loaf pre-ferments	Mild	Mild, acceptable
Aerobacter aerogenes	Acceptable, like standard	Mild, acceptable
Bacillus coagulans	Acceptable, stronger than standard	Mild, acceptable
A. aerogenes	Acceptable, faint	Mild, acceptable
B. laterosporus	Acceptable, slightly stronger than standard	Mild, acceptable
Lactobacillus plantarum	Acceptable, strong fermentationlike	Mild, acceptable
Micrococcus caseolyticus	Acceptable, strong fermentationlike	Mild, acceptable
L. leichmannii	Acceptable, mild fermentationlike	Excellent, acceptable
L. casei	Acceptable, mild fermentationlike	Mild, acceptable
A. aerogenes plus *L. casei*	Acceptable, stronger than standard	.....
L. casei plus *laterosporus*	Acceptable, more acid than *B. laterosporus* alone	Acceptable
Aged sponge dough		
L. fermenti	Mild, like standard	Mild, acceptable
L. casei	Mild, like standard	Mild, acceptable
L. brevis	Cheeselike	Bad, not acceptable
L. lactis	Not acceptable, strong	Not acceptable
L. bulgaricus	Cheeselike	Faint acid
L. plantarum	Cheeselike	Faint acid
Lactobacillus sp.	Not acceptable, strong	Not acceptable
Dairy bacterial cultures		
Sugar-3 *(L. plantarum)*	Mild, acceptable	Mild, acceptable
Old buttermilk plus *L. brevis* F 118	Mild, acceptable	Mild, acceptable
L. brevis F 118	Mild, acceptable	Mild, not acceptable
Buttermilk culture	Mild, acceptable	Mild, acceptable
Buttermilk culture plus *L. brevis* F 118	Mild, acceptable	Mild, acceptable
Sugar-2 *(L. bulgaricus)*	Mild, acceptable	Mild, acceptable
L. bulgaricus 09	Mild, acceptable	Mild, acceptable
Buttermilk cultures plus *L. bulgaricus* 09	Mild, acceptable	Mild, acceptable
L. delbrückii LD 5	Mild, acceptable	Mild, acceptable
Buttermilk culture plus *L. delbrückii* LD 5	Mild, acceptable	Mild, acceptable
Old buttermilk culture	Sharp, not acceptable	Strong, not acceptable
Propionibacterium No. 1	Mild, stronger than standard	Mild
Propionibacterium No. 2	Different, sharp, not acceptable, fast fermentation	Mild, cheeselike
Lyophilized		
B. pumilus	Creamlike, slight, acceptable	Mild, different
B. laterosporus	Weaker than standard	Mild, different

the 6-hour fermentation period appeared to produce dough with increased "oven spring."

The microorganisms predominant in aged sponge dough were members of the genus *Lactobacillus*. This condition might have been influenced by fermentation time and development of acidity during fermentation. Usually in mixtures containing milk, the *Streptococcus lactis* group grows first and develops acidity which conditions the medium for the growth of lactobacilli.

The bacterial counts per gram obtained using orange serum agar, tomato-juice agar, and nutrient agar were 2,500,000, 2,000,000, and 0, respectively. Thus, orange serum agar appeared to be a better medium than either tomato-juice agar or nutrient agar for the growth of microorganisms present in sponge dough. Plates of these three media prepared from the same dilution blanks showed abundant, clear growth on the orange serum agar. There was no growth of any microorganisms on the corresponding nutrient agar plates. This was further indication that the *Lactobacillus* species were predominant in the sponge dough. The observations agree with those of Fuller (6), who found that *L. panus acidi* (see footnote 3) was predominantly present in all "leaven" samples of bread tested.

The odors of bread and toast made with selected microorganisms from sponge dough are recorded in Table III. The bread score and odors of bread and toast made with organisms from the dairy cultures also are recorded in Table III. Loaves made with *Lactobacillus bulgaricus* 09 and *L. bulgaricus* 09 plus buttermilk cultures were scored highest in this series and were judged to be acceptable with respect to flavor and odor. Bread made with the organism *L. plantarum* and buttermilk cultures plus *L. brevis* F 118 were scored second best in this series and were judged to be acceptable with respect to flavor and odor. Breads made with the above-named cultures of organism had good grain and texture which might have been influenced by the cultures.

Results of the triangle test (13, 15) made on bread produced with organisms *Lactobacillus leichmannii* and *L. plantarum* showed the flavor to be acceptable but not significantly different from that of normal bread. Bread dough made with *Propionibacterium* No. 2 fermented rapidly, but the finished product was judged to be nonacceptable in flavor and odor in the fresh state (Table III). However, the toasted product was acceptable.

Bacterial counts per gram of lyophilized *Bacillus pumilus* and *B. laterosporus* were 84,000,000 and 160,000,000, respectively. Thus, lyophilization did not affect the viability of these organisms. Lyophilization without previous centrifuging and washing, however, resulted in an

objectionable odor in the dry product. Breads made with organisms that had been centrifuged, washed, and lyophilized were judged to be acceptable and different in character from ordinary bread, whereas breads made with nonwashed lyophilized or nonlyophilized organisms were judged to be unacceptable (Table III).

The baking industry is constantly searching for ways of improving the flavor of white pan bread. These experiments suggest the possibility of using selected microorganisms in pre-ferments or in the sponge to enhance bread flavor.

Acknowledgment

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STUDIES ON THE DECREASE OF THE BACTERIAL POPULATION IN PRE-FERMENTS[1]

ROBERT J. ROBINSON, THOMAS H. LORD, JOHN A. JOHNSON, AND BYRON S. MILLER

ABSTRACT

The production of acids or alcohols, the presence of bacteriophage, lowered oxygen tension, raised carbon dioxide or raised nitrogen tension did not account for the decrease in numbers of bacteria in pre-ferments during fermentation. Two antibiotic substances were isolated in an ether extract of a concentrated inorganic salt-buffered pre-ferment. These were designated I_1 and I_2 and had R_f values on paper chromatograms of 0.24 and 0.68 respectively, with the use of a solvent containing ethanol, ammonia, and water (80:5:15, v/v). I_1 and I_2 fluoresced blue and yellow (366 mμ), respectively. They possessed inhibitory properties for *Micrococcus pyogenes* var. *aureus* L 41, *Escherichia coli* L 145, and mixed pre-ferment cultures. Experiments indicated that the decrease in bacterial numbers in the pre-ferments was due to these inhibitory substances elaborated by the yeast during fermentation.

Early workers studying pre-ferments noted that the number of yeast cells did not increase with time and that a sharp decline occurred in the bacterial population. Micka (18) observed, after yeast growth reached its optimum in 10 to 15 hours in cracker sponges, that bacterial and yeast growth were retarded. Bacterial growth was retarded to a greater extent than yeast in the later stage of sponge fermentation and was favored by a low percentage of yeast.

Choi *et al.* (6) and McLaren (17) suggested that the decline in the bacterial population in pre-ferments might be due to the germicidal effect of alcohol produced during fermentation. The production of acids with time also was offered as a possible reason for the decrease in the bacterial population in pre-ferments (Choi *et al.* 6). Milk in a pre-ferment prevents large changes in pH (6,16). Johnson *et al.* (15) showed that total acidity in three different pre-ferments reached a maximum after 3 hours of fermentation and then gradually declined. Barton-Wright (3) noted that when flour was dampened to 18% moisture, the bacterial population decreased at a faster rate during storage because an increase in hydrogen-ion concentration occurred in the abnormally moist samples.

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Florey *et al.* (10) reported on the antagonism of yeast to bacteria and molds. They noted that antibiotic substances isolated from yeast inhibited the development of members of the genera *Bacillus, Corynebacterium, Escherichia, Aerobacter, Streptococcus, Micrococcus, Proteus, Pseudomonas, Salmonella, Diplococcus,* large viruses, and others.

Haydück (13) obtained a volatile thermolabile toxic extract from yeast which he believed to be an amine. This observation was confirmed by Fernbach (8, 9). Growth of *Escherichia coli*[2] and staphylococci was inhibited (9). Guilliermond (12) also believed that the toxic substance was an amine. Schiller (22) demonstrated, by means of forced antagonism, the presence of an inhibitory substance in yeast which he believed was an enzyme since it appeared to lyse the staphylococci. Although Henrici (14) reported that yeast contained no demonstrable antibacterial activity, Bachmann and Ogait (1) believed that the inhibitory action of bakers' yeast was due to the production of acetaldehyde.

Barglowski (2) found that the addition of 50% of the filtered culture fluid from two strains of *Saccharomyces cerevisiae* or from eight out of ten strains of *Mycotorula albicans* suppressed the growth of the tubercle bacillus *(Mycobacterium tuberculosis)* but had only a slight effect on the timothy-grass bacillus *(Mycobacterium phlei).* Gilles (11) demonstrated an antagonistic principle in yeast by growing yeast and *Penicillium glaucum* together. The active principle was present in the filtrate and was destroyed by aging, drying, or radiation with ultraviolet light.

Cook *et al.* (7) prepared an antibiotic from bakers' yeast which inhibited the growth of *Asperigillus niger* and *Penicillium glabrum,* but which did not retard the growth of *Escherichia coli* or *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*). However, morphological changes of bacteria and fungi were observed. The active agent was nonvolatile, readily soluble in 95% alcohol and acetone containing a trace of water, and stable at 100°C. at pH 7.3.

By autolyzing and autoclaving commercial brewers' and bakers' yeasts, Takahashi (23) extracted substances that inactivated the tobacco mosaic virus. These substances were precipitated from solution by acetone or alcohol, were heat-stable, and were not destroyed by trypsin or absorbed by heat-denatured virus. Tikka and Itkonen (24) stated that bakers' yeast grown in rye decoction produced thermolabile enzymes that were strongly bactericidal to *Aerobacter aerogenes.*

Carpenter (5) reported on the antibiotic action of *Torulopsis utilis*

[2] The terminology for all bacteria is that used in reference 4. When an author has used an older name it appears first, followed by the Bergey name in parentheses.

and some true yeasts. He concluded that the antibiotic action of these organisms on solid media against *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*), *Pseudomonas pyocyaneus*, and *E. coli* was due to the development of acid.

Sartory and Meyer (21) obtained from a plasmolysate of bakers' yeast a potent inhibitor for *Escherichia coli* and *Proteus vulgaris*. They concluded that only fresh yeast cultures contained the toxic substance, since extracts of dry yeast by the same method proved ineffective. Uroma and Virtanen (26) isolated an oily antibacterial substance from crushed yeast cells. The material was believed to be a mixture of unsaturated fatty acids, because the inhibition was similar to that obtained with unsaturated fatty acids extracted from linseed oil. Toda (25), repeating the experiments of Schiller (22), found complete inhibition of *Penicillium glaucum* and *Salmonella typhosa* cultures by yeast. It is possible that Toda (25) was dealing with fatty acids.

Motzel (19) developed a procedure for isolating inhibitory substances from yeast. Two inhibitory substances isolated were colorless and acidic in reaction. Comparative ultraviolet and infrared spectrograms indicated that the two substances might be peptides. Chromatograms of the hydrolysates of these substances supported this hypothesis, by revealing the presence of several amino acids.

The purpose of this research was to determine the cause of the decrease in bacterial numbers in pre-ferments.

Materials and Methods

Pre-Ferment Variations. The pre-ferments used were prepared as described by Robinson *et al.* (20).

Determination of pH and Total Acidity of Pre-Ferments. The pH of each pre-ferment was determined at 1- or 2-hour intervals using a Beckman Model GS pH meter. The total acidity was determined electrometrically in 25-ml. aliquots of each pre-ferment, using 0.1*N* sodium hydroxide and a Beckman automatic titrimeter. The titration end-point of pH 8.0 was reached within 2 minutes after the neutralization was started.

Aeration of Pre-Ferments. Each pre-ferment was divided into two equal portions, one of which was placed in a flask prepared with glass tubing connected to a tank of any one of the compressed gases (nitrogen, carbon dioxide, or oxygen). All flasks were incubated at 30°C. for 24 hours during sparging. The other portion of each pre-ferment was placed in a flask without an aeration device and incubated at 30°C. for 24 hours.

Preparation of Yeast Extracts and Distillates. Twenty grams of com-

pressed yeast dried at 18°C. for 30 hours or commercial active dry yeast[3] were extracted by the method of Haydück (13). A 500-ml. portion of each extract was filtered, centrifuged, neutralized to pH 7 with 0.1*N* sodium hydroxide, and sterilized by filtering through a Seitz filter. The remaining portions of the extracts were adjusted to pH 9.0 with sodium hydroxide. One-half of each portion was distilled under reduced pressure at 40°C.; the remaining portion was distilled under atmospheric pressure. Each distillate was collected in a flask containing 1 ml. of 1*N* sulfuric acid and later neutralized to pH 7 with sodium hydroxide. The distillates were sterilized by filtering through Seitz filters and stored in the refrigerator until tested.

Testing Yeast Extracts and Distillates for Inhibition. Each 100-ml. portion of the vacuum distillate, vacuum residue, boiled distillate, or boiled residue from the yeast was inoculated with *Micrococcus pyogenes* var. *aureus* L 41 and *Escherichia coli* L 145, respectively. The number of cells was determined by use of a nephelometer and the final concentration adjusted to 1 million cells per ml. The inoculated liquids were incubated at 30°C. for 24 hours. Samples were withdrawn and plated in nutrient agar after 0, 2, 4, 6, and 24 hours of incubation. The plates were counted at the end of 24 hours of incubation at 37°C. The tests were repeated with the addition of a 10% sterile sucrose solution to the inoculated liquids.

Three Pyrex dishes, 8 in. by 11 in., were sterilized and filled with 400 ml. of sterilized nutrient agar which was allowed to harden. Three bottles of sterile nutrient agar (125 ml.) were inoculated separately with *Micrococcus pyogenes* var. *aureus* L 41, *Escherichia coli* L 145, and mixed selected cultures isolated from pre-ferments. The inoculated agars were poured onto the solidified agar in the Pyrex dishes. Strips of developed chromatograms of filtrates and extracts were laid parallel and with proper spacing on top of the solidified inoculated agar. Observations were made for zones of inhibition at the end of 24 hours of incubation at 37°C.

Isolation of the Inhibitory Substances from Pre-Ferments. The preferment formula used for the isolation of the inhibitory substances is recorded below.

Ingredients	*Quantity*
	g.
Water	3840.0
Dextrose	504.0
Malted wheat flour	21.6
Sodium chloride	84.0
Compressed yeast	400.0
Brew improver[4]	25.2

[3] Red Star Yeast and Products Co., Milwaukee, Wis.
[4] Standard Brands, Inc., New York 22, New York.

The yeast concentration was increased over that used by Robinson *et al.* (20) in order to obtain a greater quantity of the inhibitor. After 6 hours at 30°C. the pre-ferment was centrifuged at 1400 r.p.m. for 10 minutes, and the supernatant liquid was evaporated to 400–500 ml. under reduced pressure at 40°C. The procedure for isolation of the antibiotic was essentially that of Motzel (19). The pre-ferment concentrate was adjusted to pH 2 with concentrated hydrochloric acid and extracted in a separatory funnel with three 250-ml. portions of diethyl ether. The extracts were combined, the remaining water layer was drawn off, and 56 g. Hyflo-Supercel[5] added to coagulate the emulsion. The ether was decanted and the remaining ether was pressed out of the solid residue. The solids were re-extracted with 150 ml. of fresh ether. The combined ether extracts were concentrated to 20 ml. and added to 100 ml. of water. After adjustment of this mixture to pH 6, the inhibitor was soluble in the aqueous phase. The aqueous solution was extracted with a 250-ml. portion of diethyl ether and the ether layer discarded. The aqueous solution was adjusted to pH 4 and the inhibitory substances extracted with two 100-ml. portions of diethyl ether which were concentrated to 10 ml. and chromatographed.

Method for Chromatographing the Inhibitory Substances from Pre-Ferments. The concentrated inhibitory substances in ether solution were spotted on Whatman No. 3MM paper. The chromatogram was developed by the ascending method with a solvent consisting of ethanol, concentrated ammonium hydroxide, and water (80:5:15, v/v). The separated components that fluoresced blue and yellow (366 $m\mu$) were eluted with ethanol. The substance that fluoresced pink was eluted with distilled water.

Method for Testing the Inhibitory Substances from Pre-Ferment. Ten milliliters of sterile nutrient agar were poured into a sterile Petri dish and allowed to solidify. Each dish was overlayered with 10 ml. of sterile nutrient agar which was previously inoculated with *Micrococcus pyogenes* var. *aureus* L 41 or another selected organism. One-centimeter, circular paper pads No. 740-E[6] for testing antibiotics were moistened with 0.1 ml. of the substance to be tested and placed on the solidified inoculated agar. Observations were made for zones of inhibition at the end of 24 hours of incubation at 37°C.

Results and Discussion

Effect of Variation in Ingredients. The total acid production as a function of time in the sugar pre-ferment did not account for the de-

[5] Fisher Scientific Co., St. Louis 18, Mo.
[6] Carl Schleicher and Schuell Co., Keene, N. H.

crease in numbers of bacteria. The presence or absence of yeast food, glucose, malted wheat flour, or sodium chloride also did not account for the decrease in numbers of bacteria. The presence or absence of yeast in the pre-ferment, however, markedly influenced the bacterial numbers (Table I). The bacterial count in pre-ferment containing yeast tended to decrease from an initial high to a low count after 6

TABLE I

NUMBER OF BACTERIA PER MILLILITER, PH, AND TITRIMETER VALUES PER 25-ML. ALIQUOT OF SUGAR PRE-FERMENT WITH 0.3 G. DIAMMONIUM HYDROGEN PHOSPHATE ADDED AND SODIUM CHLORIDE, MALT, AND ARKADY DELETED FROM THE FORMULA

PRE-FERMENT VARIATION	TEST	FERMENTATION TIME (HOURS)				
		0	2	4	6	24
No yeast added	Bacteria/ml.	3	5	120	1300	1200
	pH	7.95	7.80	7.80	7.80	7.70
	Titr.	0.35	0.20	0.35	0.45	0.55
Yeast added	Bacteria/ml.	2600	30	2	1	161,000,000
	pH	6.41	3.30	2.65	2.70	2.90
	Titr.	2.40	8.80	7.80	6.70	5.40
No yeast (pH controlled)	Bacteria/ml.	190	2500	2000	8600	380
	pH	7.80	4.20	3.00	2.70	2.60
	Titr.	...[a]	...[b]	...[c]	...	...
Yeast only	Bacteria/ml.	120	47	60	11	2,000,000
	pH	5.55	5.70	5.20	5.25	4.80
	Titr.	0.15	0.25	0.20	0.35	0.40
Glucose only	Bacteria/ml.	110	560	720	9900	50,000
	pH	8.50	8.60	8.05	7.80	8.70
	Titr.	0.00	0.00	0.05	0.05	...

[a] pH lowered to 6.4 with lactic acid.
[b] pH lowered to 3.35 with lactic acid.
[c] pH lowered to 2.65 with hydrochloric acid.

hours of fermentation. In the absence of yeast, the bacterial numbers tended to decrease only near the end of 24 hours of fermentation. It is possible that the increase in bacterial numbers in the pre-ferment containing yeast at the end of 24 hours may have been due to increase in accessory growth factors liberated from dead yeast cells, which, together with the high hydrogen-ion concentration, favored the growth of lactobacilli.

Effect of Hydrogen-Ion Concentration. When no yeast was added to the sugar pre-ferment (Table I) the bacterial count increased steadily during the first 6 hours of fermentation and then remained constant. The pH was virtually constant throughout the 24-hour period. When yeast was added, the bacterial count decreased sharply within 2 hours. Although one might assume that the increase in hydrogen-ion concentration may have been responsible, it is seen that when the pH of a pre-ferment containing no yeast was controlled by the addition of

acid, the bacterial count increased. This observation is confirmed by the data in Table II. Thus it was concluded that the presence of the

TABLE II

NUMBER OF BACTERIA PER MILLILITER IN THREE PRE-FERMENTS WITH AND WITHOUT SPARGING BY DIFFERENT GASES

PRE-FERMENT	FERMENTATION TIME	UNTREATED		SPARGING					
				With Oxygen		With Carbon Dioxide		With Nitrogen	
		pH	Bacteria[a]	pH	Bacteria[a]	pH	Bacteria[a]	pH	Bacteria[a]
	hours		%		%		%		%
Sugar	0	5.2	100	4.6	100	4.3	100	3.8	100
	2	3.0	55	2.4	2	2.5	87	2.2	17
	4	2.6	13	2.2	2	2.5	10	2.1	12
	6	2.6	1	2.0	0	2.5	3	2.1	5
	24	3.2	0.2	2.2	0	3.2	0	2.3	0
Salt-buffered	0	6.3	100	5.1	100	6.0	100	5.6	100
	2	4.5	74	4.6	33	5.0	43	4.6	121
	4	4.2	58	4.6	13	4.8	20	4.4	57
	6	4.2	34	4.6	7	4.5	3	4.5	16
	24	4.6	68	3.9	0	4.6	0	4.4	3
ADMI	0	6.1	100					6.0	100
	2	6.1	230	(Data unobtainable owing to				5.4	166
	4	5.6	18	excessive foaming during				5.5	145
	6	5.5	1	sparging)				5.4	145
	24	5.6	12					5.1	5

[a] Percent of original bacterial count.

yeast was the important factor relating to the decrease in the bacterial population. This conclusion was substantiated when only yeast and water were incubated, since in this case, the bacterial population again decreased markedly.

Effect of Sparging with Different Gases. In order to determine whether carbon dioxide tension was responsible for the decline in bacterial numbers, the pre-ferments were sparged with nitrogen and carbon dioxide. Removal of the carbon dioxide of fermentation by sparging with nitrogen or increasing the carbon dioxide tension did not alter significantly the rate of decrease in bacterial numbers. Likewise, it did not appear that oxygen was the limiting factor controlling the growth rate of bacteria, because increasing the oxygen tension did not alter significantly the rate of decrease of the bacteria. Indeed, in the instance of the sugar pre-ferment, oxygen tended to hasten the rate of decline in bacterial numbers.

Effect of Alcohol Concentration. Production of 5.71% alcohol during fermentation could not be responsible for the decreases in bacterial numbers in the pre-ferments, because the addition of as much as 8% alcohol did not affect the bacterial count observed during 24 hours of fermentation.

Effect of Bacteriophage. Repeated efforts were made to isolate bacteriophage from bacteria-free pre-ferments, using as test organisms pure cultures of 100 different bacteria previously isolated from pre-ferments. Since none was elicited, it was concluded that bacteriophage was not responsible for the decrease in bacterial numbers in pre-ferments.

Filtrates, Distillates, and Residues of Both Compressed and Dry Yeast. No inhibitory or antibiotic effect on selected organisms was shown by fresh aqueous yeast filtrate which had been sterilized by filtration. Inhibitory action, however, was produced by the filtrate of laboratory dried yeast prepared with 0.1% hydrochloric acid. This filtrate was most effective if it was in contact with organisms for a 6-hour period.

The results of the plate count series on inhibitory action of the extracts of dry yeast indicated that the boiled distilled filtrate and the boiled residue were most effective against *Micrococcus pyogenes* var. *aureus* L 41 and least effective against *Escherichia coli* L 145. The residue and filtered extract of commercial active dry yeast prepared under reduced pressure showed inhibition for *Escherichia coli* L 145, mixed pre-ferment cultures, and *Micrococcus pyogenes* var. *aureus* L 41, whereas the distillate was ineffective (Table III). These data confirm, in part, the findings of Loofbourow and Morgan (6) on growth-promoting and growth-inhibiting factors in yeasts.

TABLE III

INHIBITION OF THE GROWTH OF DIFFERENT ORGANISMS BY COMPONENTS OF EXTRACT OF COMMERCIAL DRY YEAST

ORGANISM	ORIGINAL FILTERED EXTRACT	RESIDUE AFTER DISTILLATION	VACUUM DISTILLATE
Micrococcus pyogenes var. *aureus* L 41	+[a]	+	−[b]
Escherichia coli L 145	+	+	−
Mixed cultures from pre-ferments	+	+	−

[a] Plus sign indicates inhibition.
[b] Minus sign indicates no inhibition.

Chromatograms of the extract of dry yeast did not reveal materials that had an inhibitory effect upon the organisms used. The chromatograms did not indicate the presence of amines or acetaldehyde, but there was some indication of the presence of amino acids or peptides which were not identified.

The extract of commercial active dry yeast soaked with 0.1% hydrochloric acid in the presence of 10% added sucrose was inhibitory and bacteriolytic for *Micrococcus pyogenes* var. *aureus* L 41 and mixed pre-ferment cultures at the 2-hour period as well as at the 24-hour

period. It did not appear to affect *Escherichia coli* L 145. These observations agree, in part, with the work of Haydück (13) and Fernbach (9) on inhibition of *Micrococcus pyogenes* var. *aureus* but disagree on the inhibition of *Escherichia coli*. There was further disagreement with the beliefs of Haydück (13), Guilliermond (12), and Schiller (22), who stated that the toxic moiety was an amine or enzyme. These data also did not confirm the conclusion of Bachmann and Ogait (1) that the inhibitory factor was due to the production of acetaldehyde.

The results of the inhibitory effect of the distillate of sugar, salt-buffered, and American Dry Milk Institute (ADMI) pre-ferments upon selected microorganisms showed no inhibition except in the case of the sugar pre-ferment. In this case the distillate was inhibitory to *Aerobacter aerogenes, Micrococcus candidus,* and *Bacillus coagulans.* The eighth hour of fermentation appeared to produce the most effective pre-ferment distillates. All of these results obtained with distillates from compressed or dried yeast or pre-ferments failed to explain the decrease in the bacterial population in the different pre-ferments. The active agent appeared to be associated with the residue.

Isolation of Inhibitory Substances from the Pre-Ferment. Using the salt-buffered pre-ferment with an increased amount of yeast and the procedure of Motzel (19), an inhibitory substance was extracted from the pre-ferment. *Micrococcus pyogenes* var. *aureus* L 41 was used to check the presence of the inhibitory substance (Fig. 1) which produced a zone of inhibition 5.2 cm. in diameter. Four components were separated by chromatography from the concentrated inhibitory substances in the ether extract. One of the substances was pink under natural and ultraviolet light and had an R_f of 0.50. This substance, the color of which faded with time, was eluted from the paper with water but did not possess inhibitory activity against *Micrococcus pyogenes* var. *aureus* L 41. Another substance which fluoresced bluish-white and had an R_f of 0.37 was eluted from the paper with ethanol, but the quantity obtained did not possess inhibitory activity. Two other substances having R_f's of 0.24 and 0.68 fluoresced blue and yellow, respectively, and were eluted from the paper with ethanol. These two substances possessed inhibitory activity against *Micrococcus pyogenes* var. *aureus* L 41, *Escherichia coli* L 145, and mixed cultures of organisms isolated from the pre-ferments. The inhibitory substance with an R_f value of 0.24 was designated I_1, and that with an R_f value of 0.68 was designated I_2. I_2 was the more effective of the two substances. I_2 (0.6 mg. dry material per disk) showed a zone of inhibition against *Micrococcus pyogenes* var. *aureus* L 41 of 2.75 cm., while I_1 (0.8 mg. dry material per disk) showed a zone of inhibition against

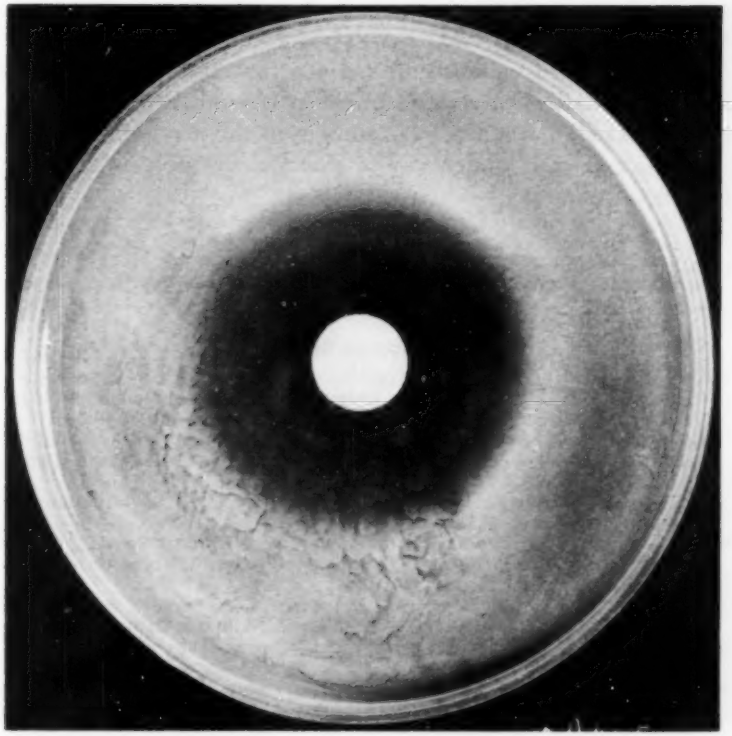

Fig. 1. Inhibition of *Micrococcus pyogenes* var. *aureus* L 41 with ether extract of pre-ferment containing two or more inhibitors.

the same organism of 1.75 cm. The combined material (0.6 mg. dry material per disk) showed a zone of inhibition against the same organism of 5.20 cm. The crude mixture of I_1 and I_2 was effective against *Escherichia coli* L 145 in liquid medium. The amount of crude active material isolated from four liters of pre-ferment was approximately 59 mg., which yielded 31 mg. of I_2 and 22 mg. of I_1 after chromatographic separation.

To determine if the inhibitory substance was produced by yeast or by bacteria, purified cultures of yeast or bacteria isolated from the salt-buffered pre-ferment were introduced into the sterile salt-buffered pre-ferment. The inhibitory substances were extracted after 6 hours of fermentation. At the same time, studies were made on the presence of an inhibitor in malted wheat flour. The results indicated that yeast was mainly responsible for the production of inhibitory substances, although bacteria and malted wheat flour also contributed to the production of antibiotic substances. The R_f values of the inhibitory

substances from bacteria and malted wheat flour were approximately 0.68, while those from yeast had R_f values of 0.24 and 0.68. Further work is required to separate the various inhibitors more completely.

Preliminary chemical studies of the partly purified inhibitory substances indicated that they were similar to those reported by Motzel (19). Further work is under way to characterize the properties of the inhibitory substances.

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VALUE OF COTTON AND JUTE FLOUR BAGS IN REDUCING INFESTATION BY THE HAIRY SPIDER BEETLE, *Ptinus villiger* (Reit.)[1]

F. L. Watters[2]

ABSTRACT

Experiments in flour storage warehouses with bags made from seven cotton fabrics and one of jute showed that finely woven, dyed, or sized fabrics reduced infestation by the hairy spider beetle, *Ptinus villiger* (Reit.), by about 90%. Sacklets made from coarsely woven cotton or jute were heavily infested. There was a high correlation ($r = +0.861$) between fabric porosity and numbers of insects per bag.

Insects commonly infest flour packed in cotton and jute bags by ovipositing through the mesh or by entering the closures and seams. In Western Canada the most serious pest of flour warehouses is the hairy spider beetle, *Ptinus villiger* (Reit.). This insect infests flour primarily by ovipositing through the fabric.

Flour manufacturers may protect bagged flour from insects by impregnating the fabric with a residual insecticide. Parkin (4) and Smallman (5) found 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT) to be effective; Cotton, Frankenfeld, and Strickland (2) recommended pyrethrins-piperonyl butoxide. However, insecticides in direct contact with flour may contaminate it; Butterfield, Parkin, and Gale (1) found that substantial quantities of DDT were transferred to flour from DDT-impregnated bags. Precautions must, therefore, be observed in the use of insecticides near foodstuffs.

[1] Manuscript received August 23, 1957. Contribution No. 3679, Entomology Division, Science Service, Department of Agriculture, Ottawa, Canada.
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A physical rather than a chemical method may be more suitable for preventing infestation of bagged flour. Although multiwall paper bags and plastic bags may protect flour from insects, bags made of these materials are more expensive than those made of cotton; also, many rural consumers prefer flour packed in cotton bags that can be reused as household articles.

A survey of flour warehouses in Manitoba showed fewer insects on closely woven bags than on bags manufactured from coarsely woven cotton or jute. It was postulated that the finely woven bags were less suitable than coarsely woven ones as sites for feeding and oviposition.

This is a report on field experiments to determine the extent to which fabrics in common use by the milling industry were infested by *P. villiger*.

Methods and Materials

Bags of 1-lb. capacity were made from seven cotton fabrics and one of jute; 25 were made from each material.

TABLE I
MEASUREMENTS OF BAG FABRICS USED

FABRIC	POROSITY INDEX [a]	THREADS PER INCH (WARP)	WEIGHT	THREAD THICKNESS
			g/sq ft	*cm* × 10^{-2}
E 21	4.45	63	13.37	2.56 ± 0.29
Print	17.50	47	12.19	2.50 ± 0.37
Bl 25	8.61	47	12.42	3.57 ± 0.99
36/3.16	16.39	48	15.28	3.29 ± 0.59
36/2.85	14.34	48	17.29	3.10 ± 0.47
Jute	17.90	14	26.23	13.22 ± 4.66
26/7.00	25.46	48	9.64	1.99 ± 0.69
31/5.00	27.60	47	11.70	2.19 ± 0.33

[a] (Area of holes/cloth area) × 100.

The fabrics are listed in Table I. Cotton fabrics are designated at the factory by the width of the roll in inches, followed by the lineal measurement in yards for 1 lb. as follows: The heaviest fabrics are 36/3.16 and 36/2.85; they are used primarily for export flour. The light-weight fabrics, 26/7.00 and 31/5.00, are used mainly for 25- and 50-lb. bags in Western Canada. Bl 25 (25/6.10) is a bleached fabric that contains a clay filling. E 21 (36/3.16) is a finely woven cotton; print (36/4.00) is a coarser cotton with various colored designs; about 1 to 5% of all 100-lb. bags sold in Western Canada comprise these two fabrics.

The fabrics were compared by the areas of their mesh openings. An enlargement negative of each fabric was projected on a screen of

tracing paper 30 in. by 21 in. The images of the openings were traced and cut out. The ratio of the weight of the cut-outs to the weight of the total projected sack area was considered to be proportional to the porosity of each fabric. This ratio multiplied by 100 was called the porosity index.

Table I gives the porosity index and other measurements of each fabric. The low porosity indexes for the closely woven fabrics E 21 and Bl 25 were probably due to the higher thread count of E 21 (63 threads per in.) and the sizing incorporated in Bl 25. The light-weight fabrics, 26/7.00 and 31/5.00, had the highest values for porosity index.

Thread thickness was measured with an ocular micrometer; ten measurements were averaged for each determination.

In April, 1955, five groups of the eight sacklets were filled with uninfested flour and placed in each of five warehouses known to be infested with *P. villiger*. They were collected in October, 1955, and sifted at the laboratory to remove all stages of the insect. An analysis of variance was applied to the data; a multiple range test described by Duncan (3) was used to find significant differences between means.

Figure 1 is a composite photograph of the fabrics.

Results

The mean numbers of spider beetles found per sacklet after 6 months in infested warehouses were:

Fabric	*No. of beetles*
E-21	7.1
Print	7.3
Bl 25	8.8
36/3.16	16.1
36/2.85	30.4
Jute	48.1
26/7.00	78.8
31/5.00	83.4

Any means within the same bracket are not significantly different at the 5% level. Any means not within the same bracket are significantly different at the 5% level.

The coefficient of correlation between fabric porosity and the mean number of spider beetles obtained from bags of each fabric was +0.861, significant at the 1% level. The standard error of estimate was 18.67.

Discussion

The results indicate that flour bags made from closely woven fabrics give good protection against infestation by *P. villiger*. Such fabrics have a low porosity index and present a physical barrier to oviposi-

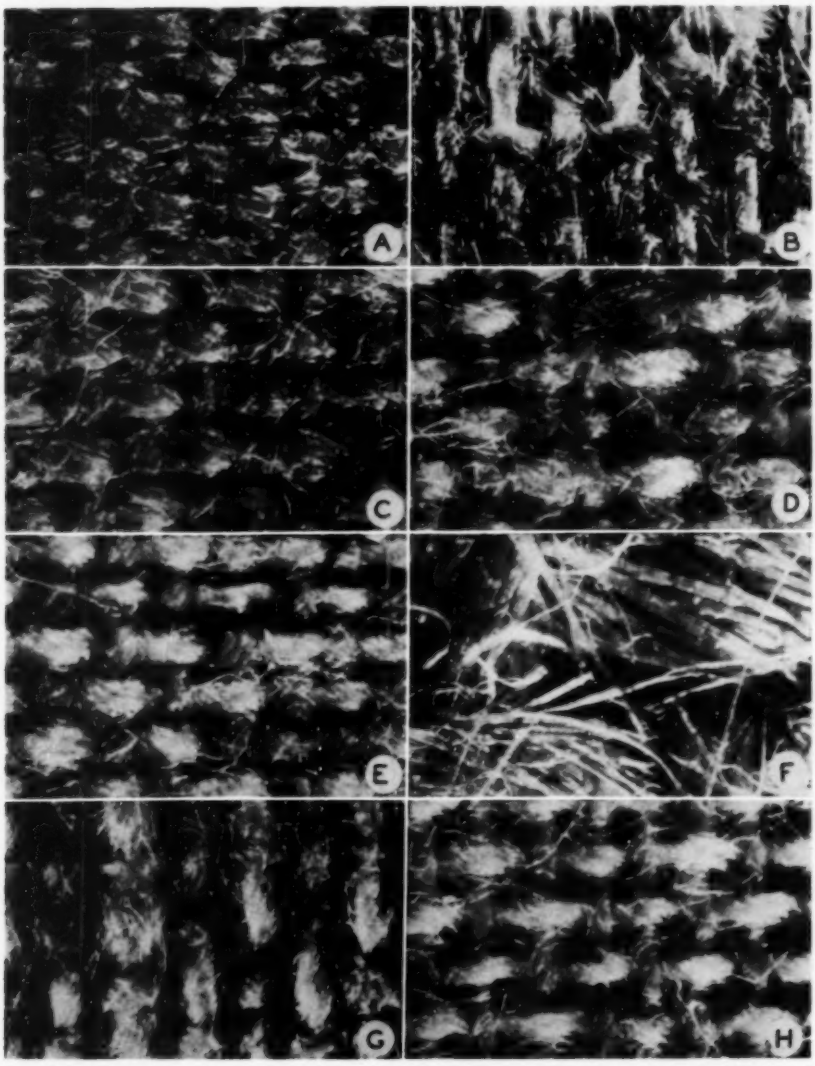

Fig. 1. Photographs of the fabrics used to make sacklets. The porosity index of each fabric is given in brackets. All but F are cotton. A, E 21 (4.45); B, print (17.50); C, Bl 25 (8.61); D, 36/3.16 (16.39); E, 36/2.85 (14.34); F, jute (17.90); G, 31/5.00 (27.60); H, 26/7.00 (25.46).

tion. Fabric porosity is related to thread thickness and the number of threads per in. The low porosity index and good protection against insects afforded by Bl 25 was due to the sizing in the fabric which served as a filler. Smallman (5) reported good protection against spider beetles when flour was packed in sized cotton bags.

The most notable result was the light infestation in sacklets made from the print fabric. Although the porosity index of print was near that of jute, significantly fewer insects were found in print than in jute sacklets. The protection against egg-laying afforded by print cannot be explained on the basis of a physical barrier; the explanation may be in the chemical nature or color of the dye used to print the fabric.

Coarsely woven fabrics are used to make most cotton or jute flour bags. The results show that bags made from these fabrics are more susceptible to insect infestation than closely woven bags. Although these results are based on experiments in flour warehouses infested with *P. villiger*, they may apply equally to other insects of similar habits. Unpublished data at the Winnipeg laboratory show that the confused flour beetle, *Tribolium confusum* Duv., and the Indian-meal moth, *Plodia interpunctella* (Hbn.), also lay eggs through cloth fabrics.

Through more widespread use of closely woven or print cotton bags, manufacturers may achieve greater protection of their products from insects during storage. This will reduce considerably the losses caused by insect infestations.

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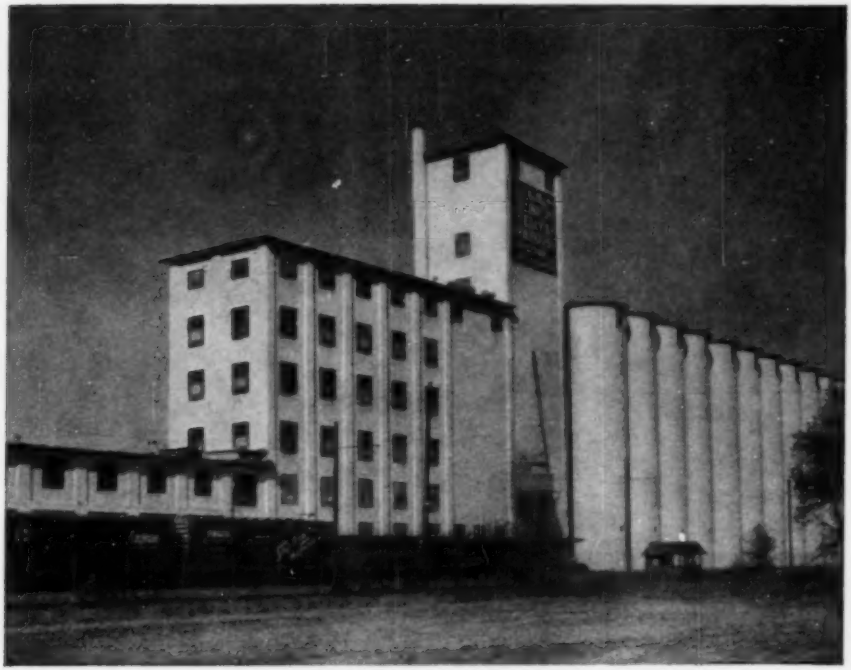

The William Kelly Milling Company of Hutchinson, Kansas, which has a daily capacity of 5000 cwt.

WHY WILLIAM KELLY MILLING CO. USES W&T FLOUR TREATMENT

The William Kelly Milling Company, with fifty years of experience, knows that the best grade of flour requires precise and uniform treatment methods. Because of this knowledge, they insure that their flour is uniformly aged, and of the best color, by using Wallace & Tiernan flour treatment. This includes: Dyox® for maturing; a Beta Chlora® unit for conditioning; and Novadelox® for bleaching. In addition, the William Kelly Milling Co. has available, at all times, skilled and experienced technical assistance from Wallace & Tiernan to aid them in the solution of their flour processing problems.

The William Kelly Milling Co. is only one of the many flour milling companies using W&T Flour Treatment. If your mill is not one of these, investigate the advantages of Wallace & Tiernan's complete flour service.

NOVADEL FLOUR SERVICE DIVISION
WALLACE & TIERNAN INCORPORATED
25 MAIN STREET, BELLEVILLE 9, NEW JERSEY
REPRESENTATIVES IN PRINCIPAL CITIES

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